Remarks

Claims 26-48 are pending in the instant application. Applicants thank the Examiner for acknowledging the allowability of claims 26-35, 37, and 44-48. Applicants respectfully address the Examiner's rejections of the remaining claims below.

I. Withdrawal of Claim 38

Applicants note that the Examiner has withdrawn claim 38 from consideration as drawn to a non-elected invention. Applicants respectfully disagree with this withdrawal.

In particular, while Applicants do not dispute that claim 38 is not within the ambit of the elected group as originally drawn by the Examiner, claim 38 is a process claim that depends from allowed product claim 26. As acknowledged by the Examiner in the office action of April 13, 2006 at page 6, pursuant to MPEP § 821.04, "[p]rocess claims that depend from or otherwise include all of the limitations of the patentable product will be entered as a matter of right if the amendment is presented prior to final rejection or allowance." As the instant office action indicated the allowance of claim 26, from which claim 38 depends, Applicants respectfully assert that claim 38 should not have been withdrawn in the action and must be rejoined and examined for patentability.

II. Objection to the Specification

The Examiner has requested that the specification be amended to reflect the status of the parent application, U.S. Patent Application No. 10/187,904. See, page 2, section 6. In accordance with the Examiner's request, Applicants have amended the first paragraph of the specification to reflect that U.S. Patent Application No. 10/187,904 has issued as U.S. Patent No. 6,683,161 on January 27, 2004.

Applicants respectfully request that the Examiner reconsider and withdraw this objection.

III. Written Description Rejections Under 35 U.S.C. § 112, First Paragraph

The Examiner has rejected claims 36 and 39-43 under 35 U.S.C. § 112, first paragraph for allegedly containing new matter. In particular, the Examiner alleges that the specification fails to provide adequate support for the recitation "an isolated cell that

produces the antibody or fragment thereof" in claim 36. See, page 3, section 10, second paragraph.

Applicants respectfully disagree and traverse this rejection.

Applicants respectfully assert that the specification need not provide written description support in exactly the same words as are used in the claims. Moreover, the Federal Circuit has recently affirmed this view by holding "[i]f lack of literal support alone were enough to support a rejection under § 112, then the statement of *In re Lukach* "...that 'the invention claimed does not have to be described *in ipsis verbis* in order to satisfy the description requirement of § 112,' is empty verbiage." See *Union Oil Co. of California v. Atlantic Richfield Co. (Unocal)*, 208 F.3d 989, 1000 (Fed. Cir. 2000). Thus, there is no requirement that the claimed invention be described *verbatim* in the specification of either the present application or in the issued patent to which the present application claims priority.

The test for the written description requirement is whether one skilled in the art could reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116 (Fed. Cir. 1991); M.P.E.P. § 2163.02. The Federal Circuit recently re-emphasized the well-settled principle of law that "[t]he written description requirement does not require the applicant 'to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed,'" *Union Oil Co. v. Atlantic Richfield Co.*, 208 F.3d 989, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000). The Court emphasized the importance of what the person of ordinary skill in the art would understand from reading the specification, rather than whether the specific embodiments had been explicitly described or exemplified.

Applicants assert that the specification conveys with reasonable clarity that Applicants were in possession of the isolated cells claimed in claim 36. Contrary to the Examiner's assertions, the disclosure in the specification is not limited to hybridoma technology utilizing splenocytes and hybridoma cells. Rather, the specification discloses that "[t]he antibodies of the invention may be prepared by any of a variety of methods." *See*, page 52, paragraph 143. In addition, the specification points to numerous publications that predate the instant invention that disclose the methods of producing antibodies. *See*, page 53, paragraph 147. Thus, the specification directs the skilled artisan

to techniques that were well-known in the art at the time of filing to produce the claimed antibodies. As the Courts have established, it is not necessary to describe that which is well-known to those of ordinary skill in the art. The MPEP states:

> The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public. *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

See, MPEP § 2164.05(a) (Emphasis added).

Applicants contend that methods of producing antibodies from isolated cells were well-known in the art at the time of filing. For example, as early as 1991, skilled artisans routinely produced antibodies through cloning and expression in mammalian cells and bacteria. *See, e.g.,* Winter, G. and C. Milstein, "Man-made antibodies," *Nature* 349:293-299 (1991), submitted herein as Exhibit A. In addition, producing antibodies through phage display technology was also well-known in the art at the time of filing. *See, e.g.,* Winter, G. et al., "Making antibodies by phage display technology," Annu. Rev. Immunol. 12:433-455 (1994). Applicants asserts that upon reading the specification and in view of the knowledge in the art, the skilled artisan could reasonably conclude that Applicants were in possession of the claimed isolated cells of claim 36 at the time of filing of the instant application. Accordingly, Applicants contend that the subject matter of claim 36 is adequately described in the specification and does not constitute new matter.

The Examiner has also alleged that the specification fails to provide support for antibodies that bind an ependymin polypeptide "at least 30 contiguous amino acid residues of SEQ ID NO:2 in length" or "at least 50 contiguous amino acid residues of SEQ ID NO:2 in length." See, page 3, section 10, paragraph 3. Applicants respectfully disagree and traverse this rejection.

Contrary to the Examiner's assertions, Applicants note that the limitation of at least 30 or 50 contiguous amino acid residues is clearly supported in the specification in the context of epitope-bearing portions of an Ependymin polypeptide. *See*, Id. In particular, the specification clearly contemplates that polypeptides of the invention include portions that are at least 30 or 50 amino acids of SEQ ID NO:2. *See*, page 46, paragraph 127.

Moreover, paragraphs 135-139 specifically discuss epitope-bearing polypeptides of the invention, and specific support is given for such polypeptides being at least 30 amino acids in length. Thus, the specification provides the literal support that the Examiner contends is lacking.

Moreover, the fact that the recitation " 'at least 30/50 contiguous amino acid' reads on a fragment that is more than 50 amino acids" does not render the description in the specification lacking. The specification clearly provides the amino acid sequence of SEQ ID NO:2. *See, e.g.*, specification at Figures 1A-1C. In addition, the claims at issue incorporate closed language such that the polypeptides of claims 39-43 consist of a fragment of the Ependymin polypeptide wherein the fragment is at least 30 or 50 amino acids in length. Thus, Applicants contend that one of skill in the art would clearly be able to recognize each and every fragment encompassed by the claims at issue. Accordingly, Applicants contend that the subject matter of claims 39-43 are adequately described in the specification and do not constitute new matter.

For the reasons set forth above, Applicants submit that claims 36 and 39-43 fully meet the written description requirements of 35 U.S.C. § 112, first paragraph, and respectfully request that the Examiner's rejection of the claims under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Conclusion

Entry of the above amendment is respectfully solicited. In view of the foregoing remarks, Applicants believe that this application is now in condition for allowance, and an early notice to that effect is urged. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicants would expedite the allowance of this application.

Finally, if there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an additional

extension of time under 37 C.F.R. § 1.136, such an extension is requested and the appropriate fee should also be charged to our Deposit Account.

Dated: October 10, 2006

Respectfully submitted,

By _____
Karen L. Carroll
Registration No.: 50,748
HUMAN GENOME SCIENCES, INC.
14200 Shady Grove Road
Rockville, Maryland 20850
(301) 315-1768

MJH/KC/pb

EXHIBIT A

Annu. Rev. Immunol. 1994. 12:433–55

MAKING ANTIBODIES BY PHAGE DISPLAY TECHNOLOGY

Greg Winter[1,2], *Andrew D. Griffiths*[1], *Robert E. Hawkins*[1], *and Hennie R. Hoogenboom*[3]

[1]MRC Centre for Protein Engineering and [2]MRC Laboratory of Molecular Biology, Cambridge, UK [3]Cambridge Antibody Technology, The Science Park, Melbourn, Cambridgeshire

KEY WORDS: selection, repertoires, rearranged V-genes, V-gene segments, polymerase chain reaction

Abstract

Antibody fragments of predetermined binding specificity have recently been constructed from repertoires of antibody V genes, bypassing hybridoma technology and even immunization. The V gene repertoires are harvested from populations of lymphocytes, or assembled in vitro, and cloned for display of associated heavy and light chain variable domains on the surface of filamentous bacteriophage. Rare phage are selected from the repertoire by binding to antigen; soluble antibody fragments are expressed from infected bacteria; and the affinity of binding of selected antibodies is improved by mutation. The process mimics immune selection, and antibodies with many different binding specificities have been isolated from the same phage repertoire. Thus human antibody fragments have been isolated with specificities against both foreign and self antigens, including haptens, carbohydrates, secreted and cell surface proteins, viral coat proteins, and intracellular antigens from the lumen of the endoplasmic reticulum and the nucleus. Such antibodies have potential as reagents for research and in therapy.

INTRODUCTION

In the immune system, the rearrangement of the V gene segments creates a repertoire of virgin B cells, each displaying a single antibody species.

0732–0582/94/0410–0433$05.00

Cells are selected by encounter and binding of antigen, and they are triggered to differentiate to short-lived plasma cells that secrete antibody and to long-lived memory cells that persist in lymph nodes, spleen, and bone marrow. The V genes of the selected antibodies displayed on memory cells are subject to hypermutation, leading to antibodies of improved binding affinity after further selection with antigen. Thus repeated immunization leads to "affinity maturation" of the response (Figure 1). The immortalization of antigen-stimulated B cells by fusion to myeloma cells (1) taps the immune repertoire and has led to a wealth of rodent monoclonal antibodies with predefined specificity.

Technologies have been emerging for making antibodies in vitro by mimicking the selection strategies of the immune system (2–4). Repertoires of antibody fragments are displayed on the surface of filamentous bacteriophage, each displaying a single antibody species; the phage are selected by binding to antigen; and finally soluble antibody fragments are secreted from infected bacteria (Figure 1). As in the immune system, the V genes can be subjected to random mutation, and mutants may be selected with higher binding affinities. This allows the isolation of human antibody fragments of defined specificity, against both foreign and self-antigens. The technology is evolving fast (reviewed in 5–7), and here we review recent progress.

TECHNOLOGIES FOR SELECTION

Mimicking the B Cell

In the immune system, the B cell represents a self-replicating package containing the antibody genes that encode the antibody displayed at its surface. Phage display mimicks the B cell. Filamentous phage was first used to display small peptides by fusion to the minor coat protein (pIII: probably three or five copies per phage particle; here illustrated with three copies) (8). Two sites of pIII were used for fusion: in the flexible spacer between the two domains of pIII (8), or close to the N-terminus (9) or at the N-terminus (10). The phage were enriched by binding of peptide to monoclonal antibody. Through growth of the enriched phage and further selection by binding to antibody, very rare phage could be isolated (8).

Surprisingly, folded antibody fragments (2) and other proteins (11, 12) can also be displayed on phage. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer (13, 14), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the periplasm (2, 15–18). When antibody fragments are fused to the N-terminus of pIII, the phage is infective (2, 15). However, if the

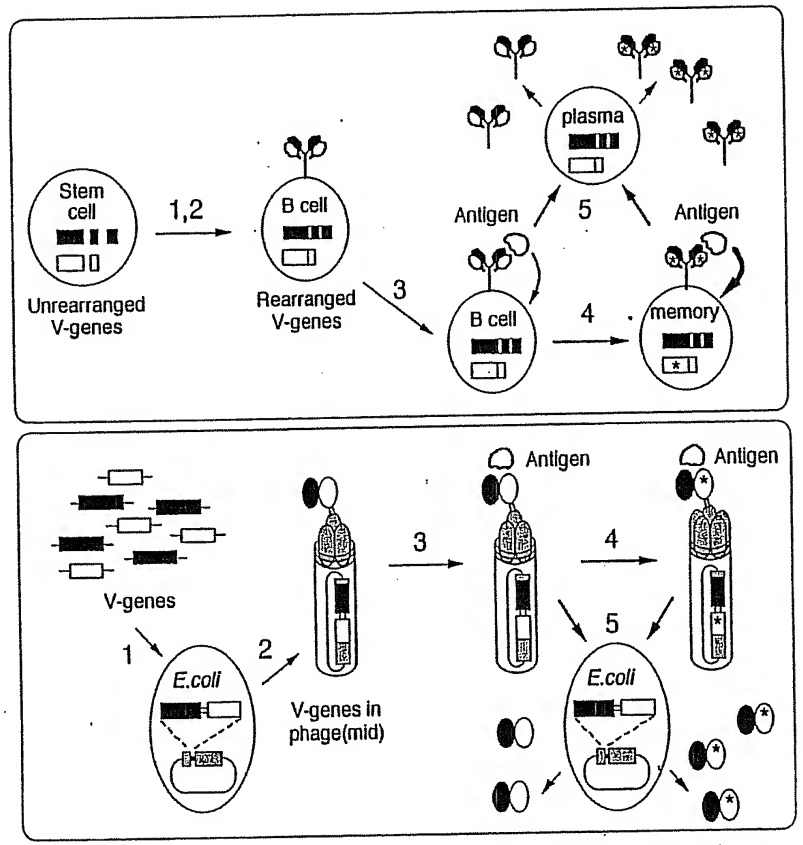


Figure 1 Generation of antibodies by the immune system and phage technology. Steps: (1) rearrangement or assembly of germline V genes; (2) surface display of antibody (fragment); (3) antigen-driven or affinity selection; (4) affinity maturation; (5) production of soluble antibody (fragment).

N-terminal domain of pIII is excised and fusions made to the second domain, the phage is not infective, and wild type pIII must be provided by helper phage (see below) (11, 16, 17) (Figure 2).

The pIII fusion and other proteins of the phage can be encoded entirely within the same phage replicon (2, 8), or on different replicons (11, 15–19). When two replicons are used, the pIII fusion is encoded on a phagemid, a plasmid containing a phage origin of replication. Phagemids can be packaged into phage particles by "rescue" with a helper phage such as

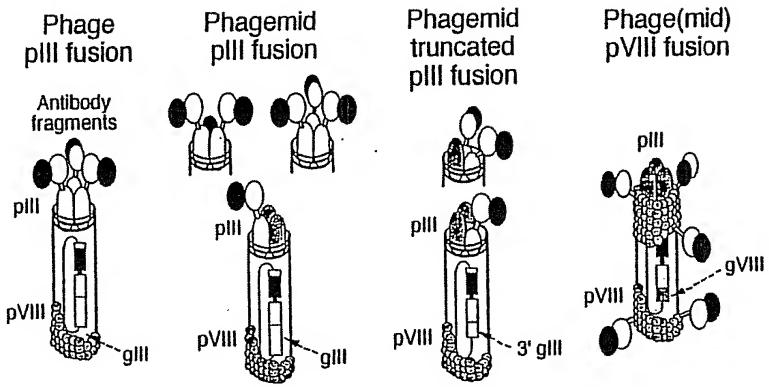


Figure 2 Display of antibody domains as pIII and pVIII fusions using phage and phagemid vectors. Antibody domains are depicted as black (heavy chain, VH or VHCH1) or white (light chain, VL or VLCL) spheroids; the genes are marked in similar fashion. Depicted are pIII fusion as phage (2) or phagemid (15) (18); truncated pIII fusion as phagemid (16, 17); pVIII fusion as phage (28, 30) or phagemid (27, 29). Only infectious phage particles displaying antibody domains are shown.

M13K07 that provides all the phage proteins, including pIII, but due to a defective origin is itself poorly packaged in competition with the phagemids (20).

The pIII fusion is often proteolysed, as shown by gel electrophoresis of the phage proteins and detection with anti-pIII antisera (J McCafferty, unpublished data). This is expected to give a population of phage particles, each displaying zero, one, two, three (and perhaps four and five) antibody fragments. The average valency of the population is further reduced by use of helper phage, in which the helper pIII competes for incorporation into the phage particle. Such phage have been estimated on average to display less than a single fusion protein per particle; they have been termed "monovalent" phage (17, 21). Other helper phages (M13ΔgIII) that lack pIII have been designed to rescue phage particles that incorporate only the pIII fusion from the phagemid; these are therefore multivalent (22). Use of different helpers can thereby alter the valency of the phages.

The major coat protein of the phage (pVIII: 3000 copies per phage particle) can also be used to display peptides (23–26) and antibody fragments (27–30). Pentapeptides (23, 24) and hexapeptides (25) were fused close to the N-terminus of pVIII, but phage encoding longer peptides were not viable unless wild type pVIII was provided (25, 26). The phage population is multivalent. With helper pVIII, up to about 900 peptides (25) and 24 antibody fragments (27) are incorporated per phage particle.

Fusions to pIII rather than pVIII have to date been preferred for antibody display.

Mimicking Immune Selection

In the immune system, encounter with antigen involves triggering the B cell through its receptor, and proliferation and differentiation to produce plasma cells that secrete antibody (reviewed in 31). The process appears capable of selecting one or more B cells from repertoires of $< 5 \times 10^8$ cells in mice and $< 10^{12}$ cells in humans (for review, see 32). Furthermore the immune system is able to selectively enrich for B cells displaying antibodies with slightly improved binding affinities, allowing affinities to be built up in a step-wise manner through rounds of mutation and selection (33).

Phage selection appears to be at least as powerful as immune selection. Phage displaying small peptides can be selected by direct binding to solid phase antibody (8), and also by binding to a biotinylated antibody in solution, which is then captured onto solid phase streptavidin (9). Likewise phages displaying antibodies can be selected by binding to antigen coated plates (16, 34), column matrices (2), cells (35), or to biotinylated antigen in solution followed by capture (36). The phages bound to the solid phase are washed and then eluted by soluble hapten (37), acid (16) or alkali (34). Phages can be enriched 20–1000 fold by a single round of selection (2, 17, 34). Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection. In this way, enrichment factors of only 50-fold in each round can build up to 10^7-fold enrichments over four rounds of selection (34).

SELECTION EFFICIENCY The efficiency of selection is likely to depend on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with (solid phase) antigen. For example, antibodies with fast dissociation kinetics (and weak binding affinities) should be retained by use of short washes, multivalent display and a high coating density of antigen at the solid phase. The high density should not only stabilize the phage through multivalent interactions, but favor rebinding of phage that has dissociated. Nevertheless, it appears that binding affinities (for a single antibody fragment) of 10^5 M^{-1} are barely sufficient to hold multivalent phage to solid phase (37).

Conversely the selection of antibodies with slow dissociation kinetics (and good binding affinities) should be promoted by use of long washes (11), monovalent phages (11), and a low coating density of antigen (38). In principle, phages with very high affinities ($> 10^{10}$ M^{-1}) should be difficult to elute, but a change in pH may suffice to dissociate the complex (21, 39);

the phage also survive 5M guanidine hydrochloride (M Figini, unpublished data).

DISCRIMINATION In immune selection, the virgin B cells displaying antibodies with (unwanted) self-specificities are deleted or rendered anergic (40). With phage, it has proved more difficult to deplete the repertoire, for example by preabsorption, as it is difficult to capture all the phage that can bind, and many of the phages are "bald," lacking antibody fragments due to proteolysis. Nevertheless, preabsorption (on red blood cells lacking the blood group E antigen) was used for isolation of phage specificities against the blood group E antigen (35).

As with immune selection, it is also possible to select between phage antibodies of different affinities (37), even with affinities that differ slightly (36). In the later immune response, B cells are thought to compete for limiting antigen in the germinal centres (for review, see 41). Likewise in selection of peptide phages with biotinylated antibody (9), limiting antibody was used to promote competition between the phages (42).

However, random mutation of a selected antibody is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting antigen, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess soluble biotinylated antigen, but with the antigen at a lower concentration than the target affinity constant. The phages are then captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, provided no two antibody fragments on the same phage bind to the same molecule of antigen. Using this technique, mutant phage antibodies have been selected from a great excess of phages with two- to four-fold lower affinities over many rounds of selection (36, 112).

Discrimination can be enhanced by taking advantage of dissociation kinetics. Thus for two phages dissociating from antigen with slightly different kinetics, the discrimination should increase with time due to the exponential nature of the decay. Indeed this was demonstrated by dissociation of phages from biotinylated antigen in solution (36). Using such kinetic selection, even mutant antibodies with a two-fold higher affinity could be selected from a great excess of phages with lower affinity (112). Washing of phages bound to a solid phase should also discriminate by dissociation kinetics.

Discrimination may be compromised by multivalent interactions (10, 11, 16, 21), but this will depend on the affinities, kinetics, and the selection process. Multiple interactions increase the avidity of phage binding, and slight differences in affinity between two antibodies should in fact give

rise to greater differences in avidity between the two phages, potentially enhancing discrimination. However, if the avidities became so strong that both phages bound very tightly to the solid phase antigen, discrimination would be lost, especially with low stringency washes.

Mimicking the Plasma Cell

Antibody fragments can be characterized and used as free soluble fragments or as phage. Binding can be detected by ELISA using antisera against the phage (2); the affinity of binding can be measured with soluble radioactive antigen (17); and dissociation kinetics by loss of phage from its complex with biotinylated antigen (36, 112). Furthermore, phages displaying antibody fragments can be used as reagents in Western blots, and for fluorescence staining of cells (A Nissim, unpublished data).

Phagemid vectors can also be engineered for display or for secretion of free antibody fragments from infected bacteria. By incorporating an amber stop codon between the fragment and pIII, the antibody fragments are fused to pIII and displayed when the amber codon is suppressed, and secreted when it is not (15). The growth of phage in suppressor and nonsuppressor bacteria therefore mimics respectively the surface display of antibodies on B cells, and the production of fragments from plasma cells (Figure 3). The same approach was used for display and secretion of human growth hormone (21). Less conveniently, the V genes encoding antibody fragments can be recloned for secretion (16, 37).

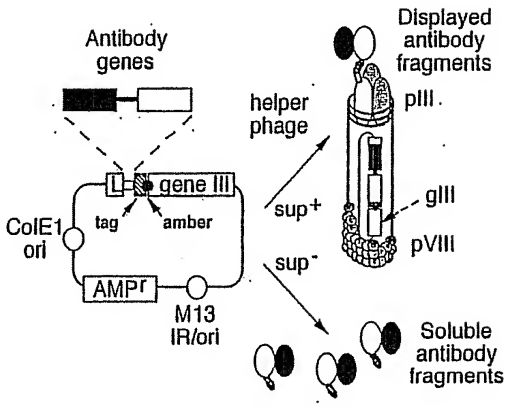


Figure 3 Mimicking the plasma cell. Phagemid pHEN1 (15) allows antibody domains to be displayed on phage after rescue with helper phage from an E. coli suppressor strain, or the domains to be secreted as (tagged) soluble fragments from non-suppressor strains. AMP = ampicillin resistance gene, L = leader peptide sequence, tag = c-myc peptide sequence.

Antibody fragments can be secreted from bacteria with yields ranging from 0.2–2 mg/l fragments in shaker flasks (43–45), or >500 mg/l in fermenters (46); and they can be harvested from the culture supernatant (44) or the periplasm (43). Protein A has been used to purify antibody fragments of the human VHIII family (47), and protein G to purify Fab fragments by binding to the CH1 domain (48). Engineered C- or N-terminal peptide tags that bind to monoclonal antibodies (49, 50) or to streptavidin (51) have also been used for both purification and detection of antibody fragments, but hexahistidine tags binding to immobilized metal chelate groups (52) seem particularly valuable for purification (53).

Antibody fragments can be characterized on a solid phase or in solution. Attempts have been made to measure binding affinities by competition ELISA (54), but the method is only qualitative (55) and may be more suitable for ranking of binding affinities. Even so this assumes no aggregation or dimerization. Thus, the reported in vitro affinity maturation of antibody fragments (56) could have been due to dimerization of the scFv fragments (57, 58). A more rigorous ELISA method (59) based on equilibrium capture would have been more suitable (55). Antibody fragments have also been characterized by binding to an antigen-coated surface by surface plasmon resonance (38, 57, 60). However, account needs to be taken of the fraction of active antibody (for determination of association rates), and of dimerization and of rebinding to the highly coated surface (for determination of dissociation rates) (61). For measurement of affinities in solution, the use of fluorescence quench titrations is often suitable for haptens (37, 62), but it is more difficult for protein antigens unless there is a large quench on binding (63).

TECHNOLOGIES FOR MAKING V-GENE REPERTOIRES

Diversity of Antibody Sequences and Structure

In the immune system the sequence diversity of antibody binding sites is not encoded directly in the germline but is assembled in a combinatorial manner from V gene segments. In human heavy chains, the first two hypervariable loops (H1 and H2) are drawn from <50 VH gene segments (64), which are combined with D segments and JH segments (65) to create the third hypervariable loop (H3). This loop is exceptionally variable in sequence and length (2–26 residues) (66); because the joining of the segments is imprecise, different reading frames of the D segment may be used, nucleotides can be inserted and deleted at the junctions, and the D segments can recombine as D-D fusions (67).

In human light chains, the first two hypervariable loops (L1 and L2) and much of the third (L3) are drawn from probably <30 Vλ (68) and <30 Vκ gene segments (JPL Cox, IM Tomlinson, unpublished data). These segments are combined with Jλ and Jκ segments to complete the third hypervariable loop (L3). This loop has limited variability. It ranges in size from 7 to 11 residues in λ light chains (69) and is most commonly 6 residues in κ light chains (70) but can vary between 5 and 8 residues (71). Thus, most of the sequence diversity (and structural diversity—see below) is encoded by the heavy chains.

Despite the immense sequence diversity, most of the loop conformations of antibody binding sites are relatively conserved (72–74). Implicit in the sequences of the VH germline segments are three major conformations for the H1 loop and five for the H2 loop. In combination they provide seven different folds (74). By contrast, the H3 loop of the rearranged heavy chains is likely to provide a huge range of structures. Implicit in the sequences of the Vλ segments are at least three major conformations for the L1 loop and at least two for the L2 loop (68). In the Vκ segments, there are probably four major conformations for the L1 loop and one for the L2 loop; in combination these provide four different folds (JPL Cox, IM Tomlinson, unpublished data). The combinations of different loops, decorated with side chains, create a wealth of binding sites ranging from flat surfaces (75) to pockets (76).

The potential diversity of different sequences in the primary immune repertoire is far greater than the number of B cells at any time. However, some sequences may not fold, and others may produce identical loop conformations: the repertoire of binding site structures is likely therefore to be much smaller than the sequence repertoire. Presumably the V gene segments and their representation in the expressed antibody repertoire reflect the efforts of the immune system over evolution to encode a diverse structural repertoire with a limited number of B cells. For phage repertoires, the V gene segments appear therefore to be suitable building blocks for making a diverse repertoire of structures.

Repertoires of VH and VL genes

The use of the polymerase chain reaction, with primers matching the 5′ and 3′ ends of rearranged VH and VL genes, provided the means to amplify, clone, and express V genes from lymphocytes (77), thereby making diverse V gene repertoires for expression (Figure 4). The V genes may be amplified from both cDNA and genomic DNA, with back primers at the 5′ end of the exon encoding the mature V-domain and forward primers based within the J-segment (49, 77). However, for amplifying from cDNA, "back" primers have also been based in the leader exon (79), and forward primers within the constant region (78). To maximize complementarity,

degeneracy was incorporated into the primers (77, 78), or different primers were designed for different families of V genes (80). For cloning of the amplified DNA into expression vectors, rare restriction sites were introduced within the PCR primer (77), as a "tag" at one end, or by further PCR amplification with a tagged primer (37). "Primary" repertoires of V genes harvested from a lymphocyte population are likely to contain somatic mutations, although most published human VH and Vκ gene sequences encode few (<5) amino acid substitutions (64; JPL Cox, IM Tomlinson, unpublished data).

Repertoires of "synthetic" rearranged V genes have also been derived in vitro from V gene segments (Figure 4). Most of the human VH-gene segments have now been cloned, sequenced (64), and mapped (81); these cloned segments (including all the major conformations of the H1 and H2 loop) have been used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length (47; A Nissim, unpublished data). VH repertoires have also been made with all the sequence diversity focussed in a long H3 loop of a single length (82). Human Vκ and Vλ segments have been cloned and sequenced (68; JPL Cox, IM Tomlinson, unpublished) and are therefore available for making synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, should encode antibodies of considerable structural diversity.

Combining VH and VL Gene Repertoires

Most of the structural diversity of antibody binding sites appears to be contributed by heavy rather than light chains (see above). Indeed, heavy

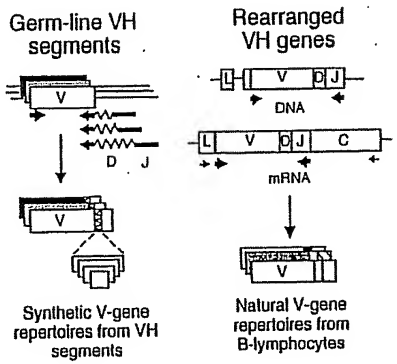


Figure 4 Generation of V gene repertoires. The location of the primers for PCR amplification of V gene repertoires from segments (47) or from rearranged V genes (34, 37) are indicated.

chains and VH domains (49) have been found with binding activities in the absence of the light chain. Furthermore in camels, two of the heavy chain isotypes lack the CH1 domain and do not appear to associate with light chains (83). However, the structures of complexes of antibody and antigen indicate that usually both domains make important interactions (75, 84–86). Presumably the role of VL domains is to add structural diversity, for example, in helping to make binding clefts, and to create a larger surface of interaction with antigen. Both features should enhance the probability of finding an antibody that binds to antigen with good affinity.

Repertoires of antibody fragments have been constructed by combining VH and VL gene repertoires together in several ways (Figure 5). Each repertoire can be created in different vectors, and the vectors recombined in vitro (87) or in vivo (88); alternatively, the repertoires may be cloned sequentially into the same vector (16) or assembled together by PCR and then cloned (37). A technique of "in-cell PCR assembly" has also been described for combining the VH and VL genes within the lymphocyte by PCR, and then cloning the repertoires of linked genes (89). Repertoires of VH domains have also been combined with a single VL gene (47, 82). The route by which repertoires are combined can dictate the structural diversity and repertoire size. For example, combining VH and VL repertoires in vivo, by combinatorial infection (88) (see below), should allow the creation of libraries of $>10^{12}$ different VH/VL combinations.

ANTIBODIES MADE FROM PHAGE DISPLAY

Taking Advantage of Immunization

Immunization leads to an increase in the number of cells making an immune response, but especially in the levels of mRNA. Resting B cells make about 100 copies of Ig mRNA per cell, whereas a hybridoma (and also presumably a plasma cell) makes about 30,000 copies (90). Spleen, lymph nodes, tonsils, and bone marrow (but not peripheral blood lymphocytes) provide a rich source of plasma cells and Ig mRNA. Repertoires of VH or VL genes amplified from the mRNA of spleen cells of an immunized mouse are therefore greatly enriched in V genes encoding part of an antigen binding site (91).

In random combinatorial libraries (48), the VH and VL gene repertoires are combined at random, and the original combinations of the immune lymphocyte are destroyed. Nevertheless, if the V gene repertoires are derived from the mRNA of lymphocytes after immunization, antigen binding fragments are created at low frequency, at best <1/500 (92), and more usually <1/5000 (93, 94). The power of phage selection allows

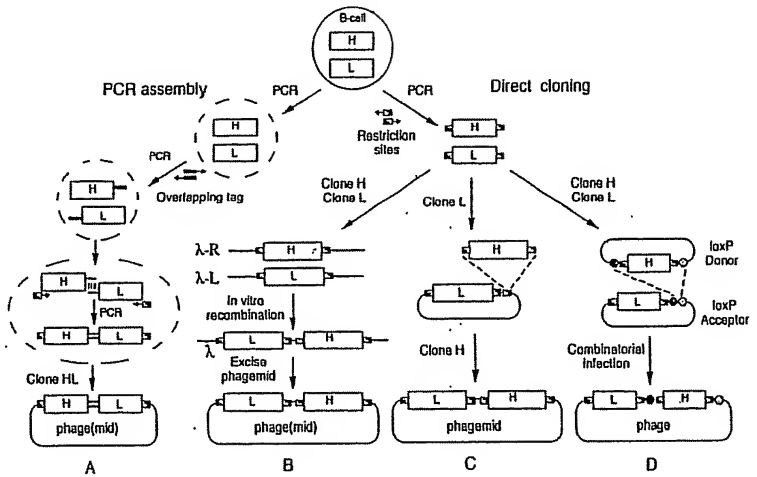


Figure 5 Linking the V genes together. (A) PCR assembly allows a one-step cloning of heavy (H) and light (L) DNA in scrambled pairings (34, 37), or original pairings if "in-cell" (89). Alternatively (B, C, D) heavy (H) and light (L) chain DNA is cloned separately and combined by in vitro recombination (B) (87), or combinatorial infection (D) (88), or cloned sequentially (C) (16).

many of these fragments to be isolated and characterized. For example, a repertoire of antibody fragments was assembled for phage display from the mRNA of mouse splenocytes after immunization with the hapten phenyloxazolone (phOx). The VH and Vκ genes encoding a range of fragments were found to be similar to those of hybridomas of the phOx response, but in general the pairings were not (37, 95).

Furthermore, as suggested by λ phage combinatorial libraries (93, 94), the pairings were promiscuous, that is, the same light chain could be found with different heavy chains, and vice versa. By "shuffling" a promiscuous heavy chain with the repertoire of light chains, a further range of partners were found for binding to phOx (37). Combinatorial repertoires from immunized sources therefore appear to be dominated by "artificial" pairings, as predicted (4). Although original pairings are likely to be present in large random combinatorial libraries, it is impossible to distinguish original from artificial pairings. However, it may be possible to determine these pairings by first linking the VH and VL genes within the lymphocyte (89)

Nevertheless, the artificial pairings from phage display libraries and, enriched by immunization, can provide antibody fragments with good affinities. For example, an antibody fragment isolated from the phOx

response (as above) had a binding affinity of 10^8 M^{-1} for hapten, with every prospect that higher affinity antibodies were present in the repertoire (37). This compares with typical affinities of 10^8 M^{-1} for secondary phOx antibodies from hybridomas (62, 96), and with affinities of 7.5×10^6–4×10^8 M^{-1} for hybridomas isolated from the same immunized spleen (95).

Antibody fragments have also been isolated from immunized humans with binding activities against several viral antigens, for example, HIV gp120 (54, 97, 98), respiratory syncytial virus (RSV) (99), and hepatitis B virus (100). The fragments against HIV and RSV were capable of neutralizing virus infection (97, 99). Furthermore, specificities against herpes simplex virus, human cytomegalovirus, varicella zoster virus, rubella, RSV, and HIV were derived from the same V gene repertoire from a patient immune to these pathogens (101). Extensive chain promiscuity has also been seen for human antibody fragments derived from combinatorial libraries directed against HIV gp 120: it was argued that the heavy chains must have arisen from antigen-specific clones in vivo (98).

By-Passing Immunization

As the display and selection of antibodies on phage mimicks immune selection, it should be possible to isolate antibody fragments of any required specificity directly from a single phage repertoire of sufficient size and diversity. Importantly, it should provide antibody specificities directed against self-antigens that are difficult to raise by immunization, owing to tolerance mechanisms.

NATURAL REPERTOIRES A diverse source of rearranged V genes was provided by human peripheral blood lymphocytes (PBLs), using "family-based" PCR primers to amplify each of the human VH, Vκ, and Vλ families (80). The repertoires of VH and VL genes were combined at random, as this should destroy the original combinations and specificities of the PBLs and generate new specificities (34).

From this library, it was possible to isolate phage with binding activities against many different antigens. For example, antibodies were isolated against the foreign antigens bovine serum albumin (BSA), turkey egg lysozyme, the hapten phOx (34), and bovine thyroglobulin (57), and against the human self-antigens tumor necrosis factor α (TNFα), thyroglobulin, a monoclonal antibody, carcinoembryonic antigen (CEA), mucin and CD4 (57). Antibody fragments against the monoclonal antibody mapped to both variable and constant regions (57). Antibodies were also isolated against the human blood group antigens of the ABO and I blood group systems (B and HI), of the Rh system (D and E), and of the Kell system (Kpb) (35). For the anti-blood groups, the selections were

undertaken by binding the phage to red blood cells; the anti-E phage was only selected after first preabsorbing the phage library with red cells lacking this antigen.

The antibodies from the library were shown to be highly specific by screening for binding to a panel of other antigens (34, 57). Specificity was also demonstrated by the staining of kidney sections with the anti-B: the only cells stained were the endothelial cells bearing the blood group B antigen (35). The affinities of the antibodies were typical of a primary immune response, in the range $10^5\ M^{-1}$–$10^7\ M^{-1}$, but dimerization of the scFv fragments led to improved avidities (57). Antibody fragments were also derived from V genes prepared from unimmunized rodent bone marrow. However, the library was selected only against the hapten progesterone, the binding affinities were poor (apparently 10^4–$10^5\ M^{-1}$ by competition ELISA), and the fragments cross-reacted with another protein (56).

Although a range of anti-self specificities can be derived from a "single pot" library from "natural" rearranged V genes, it is impossible to prove that one or another of the antibody chains was not derived from B cells with self-specificity. Moreover, in most cases the sequences of both chains were somatically mutated, suggesting that the chains were derived from an antigen-driven process (35, 57); indeed and for the anti-blood group B specificities, anti-B could be detected in the donor antiserum (35).

SYNTHETIC REPERTOIRES Synthetic V gene repertoires can also be built from cloned human VH-gene segments. A repertoire (2×10^7 clones) was first constructed using a short H3 loop of five or eight random residues with each of 49 segments, and combined with a fixed light chain. Antibodies of high specificity were selected against two haptens, phOx and NIP (with affinities of up to $10^6\ M^{-1}$) and human TNF-α, but not against three other (protein) antigens (47). However, by adding a range of H3 loops of different lengths, up to 12 residues, a single library was created from which a range of more than 20 binding specificities could be selected, including against haptens; the foreign antigens lysozyme, keyhole limpet haemocyanin, streptavidin, and immunoglobulin binding protein (BIP); and the self-antigens the oncogene protein rhombotin and the tumor suppressor protein p53. The epitope of an antibody binding to p53 was mapped and found to be new. The antibodies appeared to be specific and could be used as reagents for immunofluorescence staining of p53 in the nuclei of cells, and for Western blotting of cell lysates for BIP (A Nissim, unpublished). This also illustrates that antibodies can be made against intracellular antigens, and in particular those of the lumen of the endoplasmic reticulum.

Other synthetic libraries have been built from the framework of a single

antibody. By randomizing the H3 loop a single binding specificity was selected against FITC (affinity $10^7\ M^{-1}$) (82); by randomizing the sequences of the L1, L3, H2, and H3 loops, a single binding specificity was selected against insulin-like growth factor (but not against CD4 or tissue plasminogen activator) (102). There clearly has to be sufficient structural diversity to make a working "single-pot" library.

MAKING HIGH AFFINITY ANTIBODIES

Mutation

For most purposes, antibodies must bind their antigen tightly. In the immune system, strong binding can be built from multiple weak interactions, as illustrated by the interactions of IgM with multivalent antigens such as virus. However, the higher affinity antibodies are made after repeated rounds of immunization, arising either as mutants of a primary response antibody, or as entirely new antibodies (repertoire: such antibodies may arise by somatic mutation of very low affinity antibodies (96). The increase in binding affinity of primary response antibodies is sometimes modest, with anti-NP hybridomas showing a five-fold improvement in affinity (103), or large, with anti-phOx hybridomas showing improvements of 100-fold (62). Site-directed mutagenesis of an anti-p-azophenylarsonate antibody also suggests that somatic mutation at a few sites can together contribute factors of >200 to binding affinity (104).

In phages, antibody fragments can be designed with higher binding avidities, for example, as single chain dimers (57) or "diabodies" (58). Presumably other multimeric fragments could be designed to mimic IgM. Furthermore, mutation can be introduced at random in vitro (36, 56) by using error prone polymerase (105), or in vivo by use of mutator strains of bacteria (106, 107), and the phage can be selected for higher affinities. However, the affinities of antibody fragments against a hapten and a protein antigen could be improved only a modest four-fold to $10^8\ M^{-1}$ and $10^9\ M^{-1}$, respectively, using a single round of random mutation followed by multiple rounds of selection (36; RE Hawkins, SJ Russell, unpublished data). To make higher affinity mutants, it might be desirable to increase the frequency of random mutation or to combine rounds of mutation and selection, for example, by growing phage in bacterial mutator strains. Alternatively, it might be desirable to start with lower affinity antibodies (as may occur in repertoire shift), in the event that a higher affinity binding site is trapped at a local optimum and incapable of further affinity maturation (108).

Phage display appears to have potential advantages over the immune system for the creation of secondary (mutated) repertoires. Firstly, the size

of the secondary repertoires can be much larger than in immune systems. Secondly, random mutation can be focussed to the antigen binding loops or outside, for example, at framework residues that influence loop conformation (63). Indeed, mutations outside the contact surface with antigen can often have profound effects on binding affinity (104, 109, 112).

Chain Shuffling

In the immune system, somatic mutation of a selected pair of VH and VL domains appears to be the only mechanism for making structural variation of a selected antigen binding site. However, random combinatorial repertoires contain immense untapped diversity that can be mobilized by chain shuffling.

Chain shuffling was first used to analyze the promiscuity of VH and VL pairings in repertoires from immunized mice (37, 110). It was then used for the affinity maturation of a human antibody fragment (affinity 3×10^6 M^{-1}) for phOx isolated from a V gene repertoire. The VH gene was paired with VL genes from the original repertoire, and the new (light chain shuffled) repertoire was displayed on phage. A light chain partner was isolated that conferred improved binding affinity (6×10^7 M^{-1}). Likewise the new VL gene was paired with the original repertoire of VH genes, (but now combined with the H3 loop of the original VH gene), and after selection a fragment was isolated with a further improved affinity (10^9 M^{-1}). Indeed the affinities of the original and shuffled fragments are similar to those of mouse hybridomas of the primary and later responses to the same hapten.

In the high affinity fragment, both domains were derived from the same germline VH and VL genes as the parent, but with different patterns of mutations. The 20-residue changes suggest that large changes in affinity (500-fold here) might require many random mutations (38).

Chain shuffling can therefore be used to tap the somatically mutated V genes and make higher affinity binding sites. However, chain shuffling can also be used for more extensive diversification. For example, the heavy and light chains of mouse monoclonal antibodies against the hapten phOx (M Figini, unpublished) and human TNFα (H Hoogenboom, L Jespers, unpublished data) were sequentially replaced to create entirely human antibodies of the same specificity, a process termed *epitope imprinted selection*.

Large Repertoires

Theoretical studies have suggested, not surprisingly, that the larger the library, the greater the chance of finding antibodies that bind to any given epitope, and the higher the affinity (111). However, the limiting factor in making large primary libraries is the efficiency of introduction of plasmid or phage DNA into bacteria. In practice, this limits the library size to 10^7–10^9 clones, even taking advantage of λ phage vectors with excisable filamentous phage replicons (87).

In principle, a simple way of increasing library size would be to generate more of the possible chain combinations. This has prompted a new approach—combinatorial infection (88). For example, if 10^5 different light chains were cloned for display as a Fab-pIII fusion in a phage vector, and then the phage used to infect $>10^{12}$ bacteria harboring a library of 10^7 different heavy chains in a plasmid, this could create 10^{12} possible Fab fragments (15). If the two chains were recombined efficiently in vivo onto the same phage replicon by use of loxP sites (88), this would create a phage library with huge diversity. Indeed, it appears that such huge "teraphage" libraries can be created (88; AD Griffiths, P Waterhouse, unpublished), and this should allow high affinity antibodies to be isolated directly and indeed might also facilitate any chain shuffling required for further affinity improvements.

CONCLUSION

Phage display should facilitate the construction of human antibodies of therapeutic value and of research reagents. Libraries have been constructed that take advantage of immunization, or by-pass it, leading to antibodies with good binding affinities (10^8–10^9 M^{-1}) and high specificity against foreign and self-antigens. Targets have included viral coat proteins, BIP from the lumen of the endoplasmic reticulum, and surface markers of lymphocytes (T cell receptor and CD4), tumor cells (CEA and mucin) and red blood cells (B, D, E, I and Kell). The antibodies have been used to neutralize virus, to stain cells, and for Western blots.

There is clearly a future for "single pot" libraries, as the same library can be selected with a range of different antigens, and without the need for immunization of animals. The affinities of the antibodies isolated should improve as new technologies are used to increase the size and diversity of libraries. Indeed, the availability of the cloned human VH, Vκ, and Vλ gene segments, and knowledge about the structures they encode, should allow the design of maximum structural diversity in primary repertoires. It should also allow the creation of premutated genes for use in making secondary repertoires, in which mutations are focussed at the antigen contacts or at sites likely to modulate the contacts.

There may also be a future for "designer" libraries. As the potential antibody diversity is probably too large to be tapped in a single phage library, it may be advantageous to build libraries that are shaped for

complementarity to a defined antigen. As phage display can not only exploit the principles of immune selection, but also cannibalize and improve on the antibody building blocks, it should increasingly be capable of outperforming natural immune systems in making antibodies.

Literature Cited

1. Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–97
2. McCafferty J, Griffiths AD, Winter G, Chiswell DJ. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348: 552–54
3. Milstein C. 1990. The Croonian lecture, 1989. Antibodies: a paradigm for the biology of molecular recognition. *Proc. R. Soc. Lond. Biol.* 239: 1–16
4. Winter G, Milstein C. 1991. Man-made antibodies. *Nature* 349: 293–9
5. Marks JD, Hoogenboom, HR, Griffiths, AD, Winter G. 1992. Molecular evolution of proteins on filamentous phage: mimicking the strategy of the immune system. *J. Biol. Chem.* 267: 1–4
6. Hoogenboom HR, Marks JD, Griffiths AD, Winter G. 1992. Building antibodies from their genes. *Immunol. Rev.* 130: 41–68
7. Griffiths AD. 1993. Production of human antibodies using bacteriophage. *Curr. Op. Immunol.* 5: 263–67
8. Smith GP. 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315–17
9. Parmley SF, Smith GP. 1988. Antibody-selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* 73: 305–18
10. Cwirla SE, Peters EA, Barrett RW, Dower WJ. 1990. Peptides on phage: a vast library of peptides for identifying ligands. *Proc. Natl. Acad. Sci. USA* 87: 6378–82
11. Bass S, Greene R, Wells JA. 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins* 8: 309–14
12. McCafferty J, Jackson RH, Chiswell DJ. 1992. Phage-enzymes: expression and affinity chromatography of functional alkaline phosphatase on the surface of bacteriophage. *Protein Eng.* 4: 955–61
13. Huston JS, Levinson D, Mudgett HM, Tai MS, Novotny J, Margolies MN, Ridge RJ, Bruccoleri RE, Haber E, Crea R, Opperman H. 1988. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in Escherichia coli. *Proc. Natl. Acad. Sci. USA* 85: 5879–83
14. Bird RE, Hardman KD, Jacobson JW, Johnson S, Kaufman BM, Lee SM, Lee T, Pope SH, Riordan GS, Whitlow M. 1988. Single-chain antigen-binding proteins. *Science* 242: 423–26
15. Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P, Winter G. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res.* 19: 4133–37
16. Barbas CF, Kang AS, Lerner RA, Benkovic SJ. 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* 88: 7978–82
17. Garrard LJ, Yang M, O'Connell MP, Kelley RF, Henner DJ. 1991. Fab assembly and enrichment in a monovalent phage display system. *Bio/Technology* 9: 1373–77
18. Breitling SD, Seehaus T, Klewinghaus I, Little M. 1991. A surface expression vector for antibody screening. *Gene* 104: 147–53
19. Collet TA, Roben P, O'Kennedy R, Barbas CF. 1992. A binary plasmid system for shuffling combinatorial antibody libraries. *Proc. Natl. Acad. Sci. USA* 89: 10026–30
20. Vieira J, Messing J. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* 153: 3–11
21. Lowman HB, Bass SH, Simpson N, Wells JA. 1991. Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry* 30: 10832–38
22. Griffiths AD, Malmqvist M, Marks JD, Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC, Hoogenboom HR, Winter G. 1993. Human anti-self antibodies with high specificity from phage display libraries. *EMBO J.* 12: 725–34
23. Il'ichev AA, Minenkova OO, Tat'kov SI, Karpyshev NN, Eroshkin AM, Petrenko VA, Sandakhchiev LS. 1989. Production of a viable variant of the M13 phage with a foreign peptide inserted into the basic coat protein. *Dokl. Akad. Nauk. Sssr.* 307: 481–83
24. Il'ichev AA, Minenkova OO, Tat'kov SI, Karpyshev NN, Eroshkin AM, Ofitserov VI, Akimenko ZA, Petrenko VA, Sandakhchiev LS. 1990. The use of filamentous phage M13 in protein engineering. *Mol. Biol. Mosk.* 24: 530–35
25. Greenwood J, Willis AE, Perham RN. 1991. Multiple display of foreign peptides on a filamentous bacteriophage: Peptides from *Plasmodium falciparum* circumsporozoite protein as antigens. *J. Mol. Biol.* 220: 821–27
26. Felici F, Castagnoli L, Musacchio A, Jappelli R, Cesareni G. 1991. Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J. Mol. Biol.* 222: 301–10
27. Kang AS, Barbas CF, Janda KD, Benkovic SJ, Lerner RA. 1991. Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc. Natl. Acad. Sci. USA* 88: 4363–66
28. Huse WD. 1991. Combinatorial antibody expression libraries in filamentous phage. In *Antibody Engineering. A Practical Approach*, ed. CAK Borrebaeck, pp. 103–20. New York: Freeman
29. Chang CN, Landolfi NF, Queen C. 1991. Expression of antibody Fab domains on bacteriophage surfaces. *J. Immunol.* 147: 3610–14
30. Huse WM, Stinchcombe TJ, Glaser SM, Starr L, MacClean M, Hellström KE, Yelton DE. 1992. Application of a filamentous phage pVIII fusion protein system suitable for efficient production, screening, and mutagenesis of F(ab) antibody fragments. *J. Immunol.* 149: 3914–20
31. Tonegawa S. 1983. Somatic generation of antibody diversity. *Nature* 302: 575–81
32. Rolink A, Melchers F. 1993. Generation and regeneration of cells of the B-lymphocyte lineage. *Curr. Op. Immunol.* 5: 207–17
33. Kocks C, Rajewsky K. 1988. Stepwise intraclonal maturation of antibody affinity through somatic hypermutation. *Proc. Natl. Acad. Sci. USA* 85: 8206–10
34. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G. 1991. By-passing immunization: Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222: 581–97
35. Marks JD, Ouwehand WH, Bye JM, Finnern R, Gorick BD, Voak D, Thorpe S, Hughes-Jones NC, Winter G. 1993. Human antibody fragments specific for human blood group antigens from a phage display library. *Bio/Technology* 11: 1145–49
36. Hawkins RE, Russell SJ, Winter G. 1992. Selection of phage antibodies by binding affinity: mimicking affinity maturation. *J. Mol. Biol.* 226: 889–96
37. Clackson T, Hoogenboom HR, Griffiths AD, Winter G. 1991. Making antibody fragments using phage display libraries. *Nature* 352: 624–28
38. Marks JD, Griffiths AD, Malmqvist M, Clackson T, Bye JM, Winter G. 1992. By-passing immunization: building high affinity human antibodies by chain shuffling. *Bio/Technology* 10: 779–83
39. Roberts BL, Markland W, Ley AC, Kent RB, White DW, Guterman SK, Ladner RC. 1992. Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. *Proc. Natl. Acad. Sci. USA* 89: 2429–33
40. Nossal GJ. 1989. Immunologic tolerance: collaboration between antigen and lymphokines. *Science* 245: 147–53
41. Berek C. 1993. Somatic mutation and memory. *Curr. Opin. Immunol.* 5: 218–22
42. Scott JK, Smith GP. 1990. Searching for peptide ligands with an epitope library. *Science* 249: 386–90
43. Skerra A, Pluckthun A. 1988. Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* 240: 1038–41
44. Better M, Chang CP, Robinson RR, Horwitz AH. 1988. *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* 240: 1041–43

45. Glockshuber R, Malia M, Pfitzinger I, Pluckthun A. 1990. A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* 29: 1362–67
46. Carter P, Kelley RF, Rodrigues ML, Snedocor B, Covarrubias M, Velligan MD, Wong WLT, Rowland AM, Kotts C, Carver ME, Yang M, Bourell JH, Shepard HM, Henner D. 1992. High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* 10: 163–67
47. Hoogenboom HR, Winter G. 1992. Bypassing immunisation: human antibodies from synthetic repertoires of germ line VH-gene segments rearranged in vitro. *J. Mol. Biol.* 227: 381–88
48. Huse WD, Sastry L, Iverson SA, Kang AS, Alting MM, Burton DR, Benkovic SJ, Lerner RA. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246: 1275–81
49. Ward ES, Gussow D, Griffiths AD, Jones PT, Winter G. 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* 341: 544–46
50. Power BE, Ivancic N, Harley VR, Webster RG, Kortt AA, Irving RA, Hudson PJ. 1992. High-level temperature-induced synthesis of an antibody VH-domain in *Escherichia coli* using the PelB secretion signal. *Gene* 113: 95–99
51. Schmidt TG, Skerra A. 1993. The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. *Protein Eng.* 6: 109–22
52. Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stüber D. 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology* 6: 1321–25
53. Skerra A, Pfitzinger I, Plückthun A. 1991. The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique. *Bio/Technology* 9: 273–78
54. Burton DR, Barbas CF, Persson MA, Koenig S, Chanock RM, Lerner RA. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl. Acad. Sci. USA* 88: 10134–37
55. Goldberg ME, Djavadi-Ohaniance L. 1993. Methods for measurement of antibody/antigen affinity based on ELISA and RIA. *Curr. Opin. Immunol.* 5: 278–81
56. Gram H, Marconi L, Barbas CF, Collet TA, Lerner RA, Kang AS. 1992. In vitro selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. USA* 89: 3576–80
57. Griffiths AD, Malmqvist M, Marks JD, Bye JM, Embleton MJ, McCafferty J, Baier M, Holliger KP, Gorick BD, Hughes-Jones NC, Hoogenboom HR, Winter G. 1993. Human anti-self antibodies with high specificities from phage display libraries. *EMBO J.* 12: 725–34
58. Holliger P, Prospero T, Winter G. 1993. Diabodies: small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA* 90: 6444–48
59. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME. 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Methods* 77: 305–19
60. Malmborg AC, Michaelsson A, Ohlin M, Jansson B, Borrebaeck CA. 1992. Real time analysis of antibody-antigen reaction kinetics. *Scand. J. Immunol.* 35: 643–50
61. Malmqvist M. 1993. Surface plasmon resonance for detection and measurement of antibody-antigen affinity and kinetics. *Curr. Opin. Immunol.* 5: 282–86
62. Foote J, Milstein C. 1991. Kinetic maturation of an immune response. *Nature* 352: 530–32
63. Foote J, Winter G. 1992. Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224: 487–99
64. Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G. 1992. The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. *J. Mol. Biol.* 227: 776–98
65. Ravetch JV, Siebenlist T, Korsmeyer S, Waldmann T, Leder P. 1981. Structure of the human immunoglobulin m locus: characterization of embryonic and rearranged J and D genes. *Cell* 27: 583–91
66. Wu TT, Johnson G, Kabat EA. 1993. Length distribution of CDRH3 in antibodies. *Proteins* 16: 1–7
67. Sanz I. 1991. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. *J. Immunol.* 147: 1720–29
68. Williams SC, Winter G. 1993. Cloning and sequencing of human immunoglobulin variable lambda gene segments. *Eur. J. Immunol.* 23: 1456–61
69. Combriato G, Klobeck HG. 1991. V lambda and J lambda-C lambda gene segments of the human immunoglobulin lambda light chain locus are separated by 14 kb and rearrange by a deletion mechanism. *Eur. J. Immunol.* 21: 1513–22
70. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. 1991. Sequences of proteins of immunological interest. 5th edit. US Dep. Health Hum. Serv., Public Health Serv. Natl. Inst. Health
71. Timmers E, Hermans MM, Kraakman ME, Hendriks RW, Schuurman RK. 1993. Diversity of immunoglobulin kappa light chain gene rearrangements and evidence for somatic mutation in V kappa IV family gene segments in X-linked agammaglobulinemia. *Eur. J. Immunol.* 23: 619–24
72. de la Paz P, Sutton BJ, Darsley MJ, Rees AR. 1986. Modelling of the combining sites of three anti-lysozyme monoclonal antibodies and of the complex between one of the antibodies and its epitope. *EMBO J.* 5: 415–25
73. Chothia C, Lesk AM. 1987. Canonical structures for the hypervariable regions of immunoglobulins. *J. Mol. Biol.* 196: 901–17
74. Chothia C, Lesk AM, Gherardi E, Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G. 1992. Structural repertoire of the human VH segments. *J. Mol. Biol.* 227: 799–17
75. Amit AG, Mariuzza RA, Phillips SE, Poljak RJ. 1986. Three-dimensional structure of an antigen-antibody complex at 2.8 A resolution. *Science* 233: 747–53
76. Alzari PM, Spinelli S, Mariuzza RA, Boulot G, Poljak RJ, Jarvis JM, Milstein C. 1990. Three-dimensional structure determination of an anti-2-phenyloxazolone antibody: the role of somatic mutation and heavy/light chain pairing in the maturation of an immune response. *EMBO J.* 9: 3807–14
77. Orlandi R, Gussow DH, Jones PT, Winter G. 1989. Cloning immunoglobulin variable domains for expression by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* 86: 3833–37
78. Sastry L, Alting MM, Huse WD, Short JM, Sorge JA, Hay BN, Janda KD, Benkovic SJ, Lerner RA. 1989. Cloning of the immunological repertoire in *Escherichia coli* for generation of monoclonal catalytic antibodies: construction of a heavy chain variable region-specific cDNA library. *Proc. Natl. Acad. Sci. USA* 86: 5728–32
79. Jones ST, Bendig M. 1991. Rapid PCR-cloning of full-length mouse immunoglobulin variable regions. *Bio/Technology* 9: 88–89
80. Marks JD, Tristrem M, Karpas A, Winter G. 1991. Oligonucleotide primers for polymerase chain reaction amplification of human immunoglobulin variable genes and design of family-specific oligonucleotide probes. *Eur. J. Immunol.* 21: 985–91
81. Matsuda F, Kyun Shin E, Nagaoka H, Matsumura R, Haino M, Fukita Y, Taka-ishi S, Imai T, Riley JH, Anand R, Soeda E, Honjo T. 1993. Structure and physical map of 64 variable segments in the 3' 0.8-megabase region of the human immunoglobulin heavy-chain locus. *Nature Gen.* 3: 88–94
82. Barbas CF, Bain JD, Hoekstra DM, Lerner RA. 1992. Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problem. *Proc. Natl. Acad. Sci. USA* 89: 4457–61
83. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, Bajyana Songa E, Bendahman N, Hamers R. 1993. Naturally occurring antibodies devoid of light chains. *Nature* 363: 446–48
84. Colman PM, Laver WG, Varghese JN, Baker AT, Tulloch PA, Air GM, Webster RG. 1987. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. *Nature* 326: 358–63
85. Stanfield RL, Fieser TM, Lerner RA, Wilson IA. 1990. Crystal structures of an antibody to a peptide and its complex with peptide antigen at 2.8 A. *Science* 248: 712–19
86. Rini JM, Stanfield RL, Stura EA, Salinas PA, Profy AT, Wilson IA. 1993. Crystal stucture of a human immunodeficiency virus type I neutralising antibody, 50.1, in complex with its V3 loop peptide antigen. *Proc. Natl. Acad. Sci. USA* 90: 6325–29
87. Hogrefe HH, Mullinax RL, Lovejoy AE, Hay BN, Sorge JA. 1993. A bac-

teriophage lambda vector for the cloning and expression of immunoglobulin Fab fragments on the surface of filamentous phage. *Gene* 128: 119–26
88. Waterhouse P, Griffiths AD, Johnson KS, Winter G. 1993. Combinatorial infection and in vivo recombination: a strategy for making large phage antibody repertoires. *Nucl. Acids Res.* 21: 2265–66
89. Embleton MJ, Gorochov G, Jones PT, Winter G. 1992. In-cell PCR from mRNA: amplifying and linking the rearranged immunoglobulin heavy and light chain V-genes within single cells. *Nucl. Acids Res.* 20: 3831–37
90. Schibler U, Marcu KB, Perry RP. 1978. The synthesis and processing of the messenger RNAs specifying heavy and light chain immunoglobulins in MPC-11 cells. *Cell* 15: 1495–509
91. Hawkins RE, Winter G. 1992. Cell selection strategies for making antibodies from variable gene libraries. *Eur. J. Immunol.* 22: 867–70.
92. Mullinax RL, Gross EA, Amberg JR, Hay BN, Hogrefe HH, Kubitz MM, Greener A, Alting MM, Ardourel D, Short JM. 1990. Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage lambda immunoexpression library. *Proc. Natl. Acad. Sci. USA* 87: 8095–99
93. Persson MAA, Caothien RH, Burton DR. 1991. Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc. Natl. Acad. Sci. USA* 88: 2432–36
94. Caton AJ, Koprowski H. 1990. Influenza virus hemagglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor. *Proc. Natl. Acad. Sci. USA* 87: 6450–54
95. Gherardi E, Milstein C. 1992. Original and artificial antibodies. *Nature* 357: 201–02
96. Berek C, Milstein C. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* 96: 23–41
97. Barbas CF, Björling E, Chiodi F, Dunlop N, Cabara D, Jones TM, Zebedee SL, Persson MAA, Nara PL, Norrby E, Burton DR. 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. *Proc. Natl. Acad. Sci. USA* 89: 9339–43
98. Barbas CF, Collet TA, Amberg W, Roben P, Binley JM, Hoekstra D, Cababa D, Jones TM, Williamson RA, Pilkington GR, Haigwood NL, Cabezas E, Satterthwaite AC, Sanz I, Burton DR. 1993. Molecular profile of an antibody response to HIV-1 as probed by combinatorial libraries. *J. Mol. Biol.* 230: 812–23
99. Barbas CF, Crowe JE, Cababa D, Jones TM, Zebedee SL, Murphy BR, Chanock RM, Burton DR. 1992. Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralise infectivity. *Proc. Natl. Acad. Sci. USA* 89: 10164–68
100. Zebedee SL, Barbas CF, Hom Y, Caothien RH, Graff R, Degraw J, Pyati J, LaPolla R, Burton DR, Lerner RA, Thornton GB. 1992. Human combinatorial antibody libraries to hepatitis B surface antigen. *Proc. Natl. Acad. Sci.* 89: 3175–79
101. Williamson RA, Burioni R, Sanna PP, Partridge LJ. 1993. Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. *Proc. Natl. Acad. Sci. USA* 90: 4141–5
102. Garrard LG, Henner DJ. 1993. Selection of an anti-IGF-1 Fab from a Fab phage library created by mutagenesis of multiple CDR loops. *Gene* 128: 103–9
103. Cumano A, Rajewsky K. 1986. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. *EMBO J.* 5: 2459–68
104. Sharon J. 1990. Structural correlates of high antibody affinity: Three engineered amino acid substitutions can increase the affinity of an anti-p-azophenylarsonate antibody 200-fold. *Proc. Natl. Acad. Sci. USA* 87: 4814–17
105. Leung DW, Chen E, Goeddel DV. 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* 1: 11–15
106. Schaaper RM. 1988. Mechanisms of mutagenesis in the *Escherichia coli* mutator mutD5: role of DNA mismatch repair. *Proc. Natl. Acad. Sci. USA* 85: 8126–30
107. Yamagishi J, Kawashima H, Matsuo N, Ohue M, Yamayoshi M, Fukui T, Kotani H, Furuta R, Nakano K, Yamada M. 1990. Mutational analysis of structure—activity relationships in human tumor necrosis factor-alpha. *Protein Eng.* 3: 713–19

108. Macken CA, Perelson AS. 1989. Protein evolution on rugged landscapes. *Proc. Natl. Acad. Sci. USA* 86: 6191–95
109. Lavoie TB, Drohan WN, Smith-Gill SJ. 1992. Experimental analysis by site-directed mutagenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysozyme. *J. Immunol.* 148: 503–13
110. Kang AS, Jones TM, Burton DR. 1991. Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc. Natl. Acad. Sci. USA* 88: 11120–23
111. Perelson AS, Oster GF. 1979. Theoretical studies of clonal selection: Minimal antibody repertoire size and reliability of self non-self discrimination. *J. Theor. Biol.* 81: 645–70
112. Hawkins RE, Russell SJ, Baier M, Winter G. 1993. The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen: The interaction of mutant DI.3 antibodies with lysozyme. *J. Mol. Biol.* In press

EXHIBIT B

Man-made antibodies

Greg Winter & César Milstein

Monoclonal antibodies can now be genetically engineered and endowed with new properties. In the future, gene technology could enable antigen-binding fragments to be made by exploiting repertoires of variable domain genes derived from immunized animals and expressed in bacteria. How readily can this approach be extended to production of '*in vitro*' repertoires of variable domain genes, and obviate the immunization of animals?

In 1975 a method was described for making cell lines that secrete a single species of antibody (monoclonal antibody) with the desired specificity to antigen[1]. The technique—'hybridoma technology'—proved to be general, and a wide range of monoclonal antibodies have been made which bind to protein, carbohydrate, nucleic acids and hapten antigens, and which even have catalytic activities[2,3], leading to many practical applications for monoclonal antibodies in research and human health-care[4-7] and to patent disputes[8]. The technology has been improved over the years, particularly by the preselection of antigen-binding B cells[9] and by screening with antigen-coated filters[10].

Hybridoma technology was first extended by somatic cell genetics, which allowed antibody mutants to be selected[11,12], their functional properties to be changed by switching heavy chain constant regions[13] and antibodies to be made with dual specificity[14]. Gene technology later revolutionized this potential as antibody genes could now be altered to order. New vistas appeared, reviving the forgotten excitement of the old discipline of immunochemistry of antibodies. Initially antibody genes were taken from hybridomas, cloned into plasmid vectors and expressed as complete antibodies in mammalian cells[15,16] or as fragments in bacteria[17-20]. The ready manipulation of the genes by cutting and pasting of restriction fragments, or by site-directed mutagenesis, has allowed the construction of new antibody reagents and fine mapping of antibody structure–function relationships.

More recently a new approach has been proposed with the potential to bypass hybridomas[21,22]. Antibody genes are cloned directly from lymphocytes of immunized animals and expressed in bacteria, and the antibody products are screened for binding to antigen[23,24]. As does hybridoma technology, the process relies on animal immunization to give rise to many antigen-specific cells. In the animal, antibodies of low affinity are first produced by antigen-induced proliferation of cells, and then higher affinity variants are generated by point mutation and selection. Hybridoma technology can immortalize these cells; gene technology can immortalize their genes. In both cases, however, it is animals that 'invent' the new molecules.

Looking ahead, can we even bypass animals and make new antibodies in vitro[25]? Two current strategies recapitulate the great immunological controversy of the 1950s—instruction versus selection[26]. The selectionists are making naive repertoires of antibody genes and selecting those with antigen-binding activity, so mimicking nature. The modern instructionists are using computer graphic techniques to build specific antigen-binding sites. Here we shall describe the manipulations that have already produced some designer antibodies of practical value, and discuss other possibilities for improving on nature.

Starting with hybridomas

The antibody (IgG) is a Y-shaped molecule, in which the domains forming the tips of the arms bind to antigen and those forming the stem are responsible for triggering effector functions that eliminate the antigen. The domain structure of the molecule makes it particularly accessible to protein engineering, as functional domains carrying antigen-binding activities (Fv, Fab fragments) or effector functions (Fc fragments) can be used separately as fragments, or swapped between antibodies[27,28] (see Fig. 1 and legend). Furthermore, the rigid β-sheet framework structure of the variable (V) domains, surmounted with antigen-binding loops, allows the transplanting of binding sites from one antibody to another[29]. These structural features have spawned a range of designer antibodies, from complete antibodies, capable of destorying pathogens or tumour cells through their natural effector mechanisms, to antigen-binding fragments that can be used to target attached toxins, or for diagnosis though bound radioisotopes (Fig. 2).

Antibodies kill cells by triggering the complement cascade at the cell surface, with consequent lysis, or by binding to receptors on the surface of specialized effector cells, such as phagocytes or killer cells and triggering phagocytosis or antibody-dependent cell-mediated cytolysis. The potential of an antibody in lysis is determined mainly by the class of the constant (C) domains (isotype). This has been dissected by making chimaeric antibodies, in which the variable domains of a rodent antibody are attached to the constant domains of human γ isotypes. The human γ1 isotype emerges as being highly effective in both complement and cell-mediated killing, and therefore the most suitable for therapeutic use against pathogens or tumour cells[30,31]. Conversely, the inactive human γ4 isotype may be more suitable for diagnostic imaging, or for blocking a damaging immune or allergic response by binding to the antigen and thus competing with destructive antibodies[32]. Analysis of the roles of individual amino-acid residues should permit the engineering of variants of a single antibody isotype with differing effector mechanisms. Thus the binding sites for the high-affinity receptor (FcRI) include the lower hinge region of the antibody[33], and the 'core' binding site for the first component of complement is a strand of β sheet in the second heavy chain constant (CH2) domain[34] (Fig. 1).

Although some rodent antibodies (particularly the mouse γ2a and rat γ2b isotypes), can trigger human effector mechanisms, they are immunogenic in human therapy. In view of the difficulties of making human monoclonal antibodies directly (see later), rodent antibodies have been 'humanized' by linking rodent variable regions and human constant regions[28] (chimaeric antibodies, Fig. 2). This reduces the immunogenicity of the antibody as shown in clinical trials[35]. But residual immunogenicity is retained (at least in part) by virtue of the foreign V-region framework[36].

A more complete way of humanizing rodent antibodies includes the replacement of the V-region framework ('reshaped' antibodies, Fig. 2). It relies on the architecture of V domains as a framework of β sheets topped with antigen-binding loops (see ref. 37 and Fig. 1). By grafting the loops, the antigen-binding site can be transferred from rodent to human antibody[29,31,38,39]. The technique was used to humanize a rat therapeutic antibody directed against mature human leukocytes[31] which proved clinically effective in destroying a large mass of tumour in two patients[40]. But reshaping requires that the different framework

regions are structurally conserved, both in the orientations of the two β sheets of each domain and in the packing of heavy chain variable (VH) and light chain variable (VL) domains together; that the hypervariable loops make the majority of contacts with antigen; and that the loops are supported in a similar way by the underlying β-sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved necessary in others. Simple molecular modelling can help identify contacts and design small changes to optimize affinities[31,41].

Although natural effector functions are powerful, antibodies can also be engineered to recruit other effector functions. For example, by generating antibodies whose antigen-binding sites have dual specificity for a target cell and an effector such as a toxin. Such 'hybrid hybridomas' are made by fusion of hybridomas of two-different specificities[14,42], each of the two

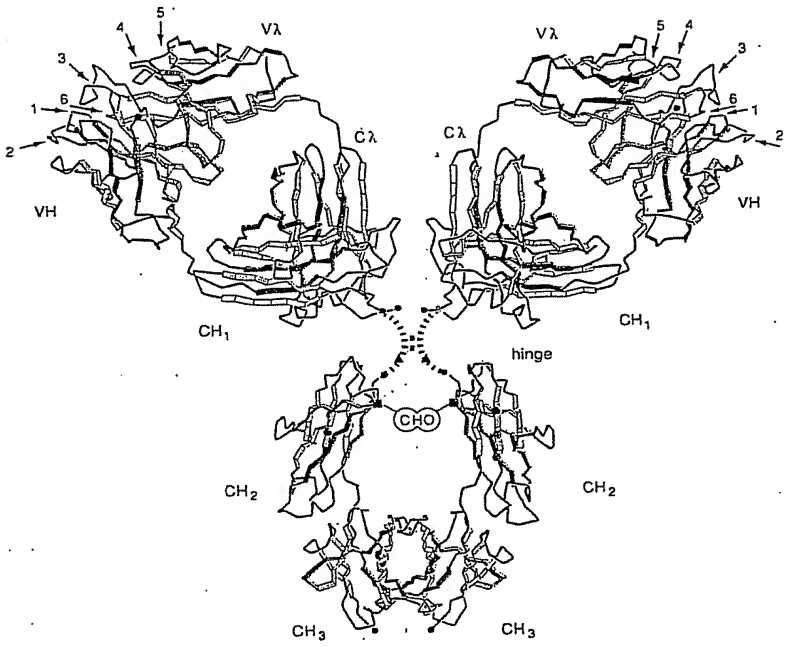
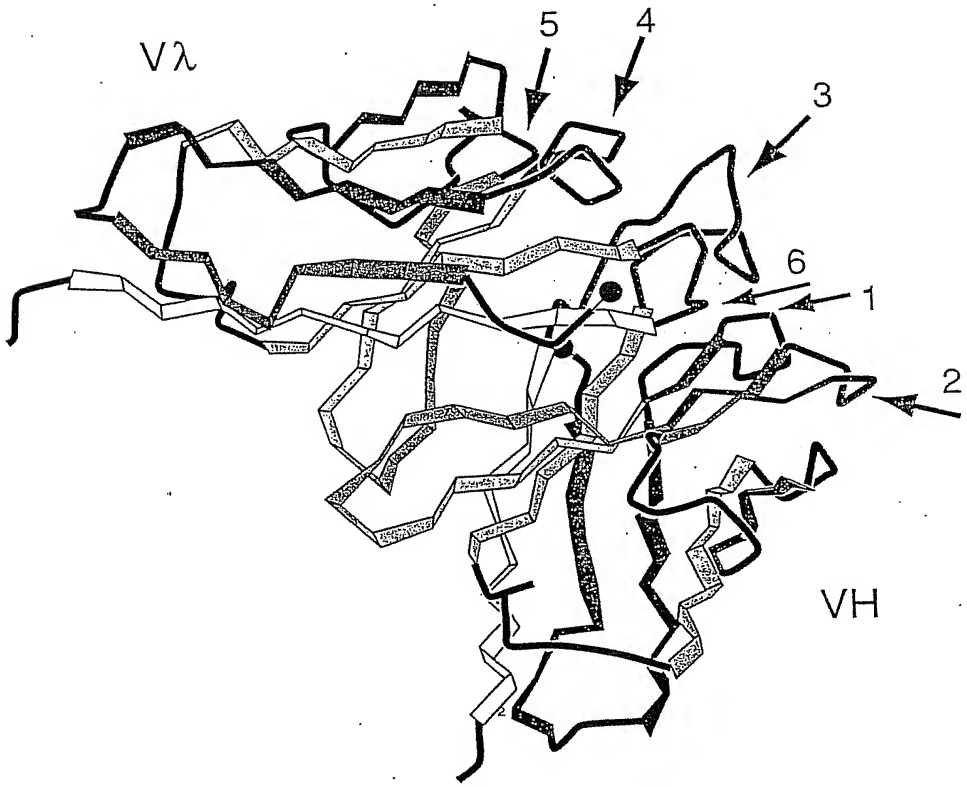


FIG. 1 Antibody structure. The antibody (IgG) consists of four chains, two heavy (H) and two light (L), which in turn are built by stringing together domains of similar architecture. Each chain is paired with another chain by lateral packing of the domains and also by at least one disulphide bond. Each domain consists of two β sheets which pack together to form a sandwich, with exposed loops at the ends of the strands. Thus the C domains have three β strands in one sheet (strands C, F, G) and four strands (strands A, B, D, E) in the other. The V domains have an extra two strands in one sheet (C, C', C", F, G). This framework is highly conserved in different antibodies. The three loops at the top of the V domain are hypervariable in sequence and fashion the antigen-binding site[37]. Despite the sequence hypervariability, most of the antigen-binding loops have a small repertoire of main-chain conformations. The antibody can be proteolytically cleaved at the flexible hinge region, yielding Fab fragments which bind antigen (comprising heavy chain VH and CH1 domains, top of the hinge and the entire light chain), and Fc fragments which bind to effector functions (comprising lower hinge and heavy chain CH2 and CH3 domains). The model is taken from the solved X-ray crystallographic structures of Fab and Fc domains of myeloma protein KOL[85], represented according to Lesk[86] and redrawn by an artist. Each strand of the β sheets has been colour-coded. The β sheet defined by the A, B, D, E strands is more intensely coloured. The hypervariable regions (VH, 1–3; VL, 4–6) are in red; the binding sites for high-affinity receptor[33], C1q[34] and carbohydrate attachment site are also marked in red, as triangles, circles or squares respectively.



halves of a single antibody molecule is from one of the two parental antibodies, and the molecule therefore carries two different antigen-binding sites. Such bispecific antibodies can bind both to a cell target (for example in a tumour) and to a toxin or cytotoxic T cell[43,44]. Novel effector functions can also be recruited by fusing gene segments encoding antigen-binding sites (as Fc or Fab fragments) to genes encoding toxins[45] and enzymes[27]. This can, for example, allow agents such as tissue plasminogen activator to be targeted to blood clots through Fab binding to fibrin, leading to the local activation of plasminogen[46]. Fusion of antibodies with enzymes may also prove invaluable for local activation of prodrugs[7,47]. Conversely antibody Fc fragments can equip other proteins with antibody effector functions. For example, CD4 linked to Fc fragments binds to viral glycoprotein gp120 on the surface of cells infected with human immunodeficiency virus (HIV), and kills the cells by antibody-dependent cell-mediated cytolysis (ref. 48) (CD4 immunoadhesin, Fig. 2).

Complete antibodies (relative molecular mass 150,000 (M_r 150K)) are large molecules but much smaller fragments can be prepared that retain antigen-binding activity. Small fragments (M_r 12K–50K) equipped with radioisotopes could be used for imaging or therapy and are in some ways particularly attractive for use *in vivo* as they penetrate tissue boundaries more effectively[49]. Fragments are also cleared faster from the serum and tissues[50] and although this may compromise their use as targeting agents, it can aid the clearance of toxic drugs, such as digoxin, from the circulation[51]. Small fragments also have advantages in fundamental research, for high-resolution X-ray crystallographic studies of antigen-binding sites. The size of antibodies and flexibility of the hinge connecting Fab arms and the Fc domain have prompted crystallographers to turn to Fab fragments[52] and now Fv fragments[53] (Fig. 2). In future we expect that fragments will be used extensively as they are readily expressed in an active form from genes introduced into mammalian[27,54] or bacterial cells[19,20,23,55]. But antibody fragments may require further engineering to eliminate undesirable properties.

For example, Fv fragments are noncovalently associated heterodimers of V_H and V_L domains which may thus dissociate. Although some Fv fragments are less prone to dissociation than others[56], stable Fv fragments can be engineered either by linking the domains with a hydrophilic and flexible peptide[57,58] to create single-chain Fv fragments (scFv, Fig. 2), or by introducing disulphide bonds between the domains[56]. Single V_H domains (dAb, Fig. 2) with antigen-binding activities[23] are likely to require more extensive engineering. The domains have an exposed hydrophobic surface (where they normally interact with light chain), rendering them 'sticky'. If their properties can be improved, for example by introducing hydrophilic residues to the interface, these single domains may prove to be the forerunners of a new generation of small recognition molecules.

Bypassing hybridomas

Immunization is essential to derive hybridomas secreting high-affinity antibodies. In the animal these antibodies are produced in two stages[59,60]. The first stage is fast and leads to the production of antibodies of low affinity (10^5–$10^7 M^{-1}$). It involves the

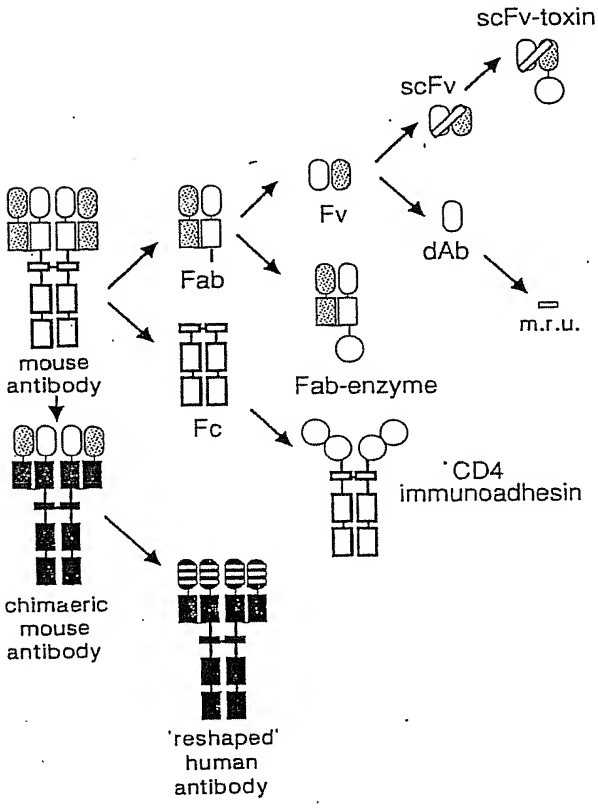


FIG. 2 Engineered antibodies and fragments. A range of engineered antibodies and fragments is depicted. Each box represents a domain. Single-chain Fv fragments (scFv), in which V_H and V_L domains are linked by a peptide (see text), and Fab fragments have been used to target enzymes and toxins. In immunoadehesins, a ligand specific for receptor (here CD4) is attached to an Fc fragment. Single V_H domains (dAbs)[23] and even single CRRs (minimal recognition units or m.r.u.)[87,88] have been identified with antigen-binding activities. Site-directed mutagenesis of antibodies and fragments has also been used to alter effector functions and improve affinities[41] (see text).

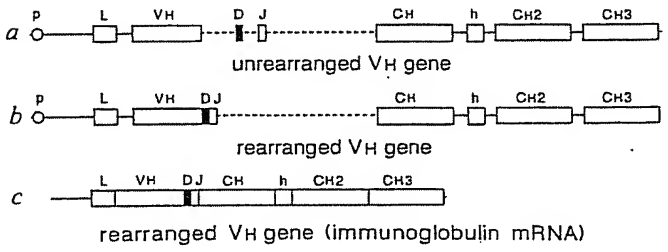


FIG. 3 Organization of V genes. The domain structure of the antibodies is mirrored at the level of the gene, as the individual domains are encoded by separate exons. In turn, the V_H and V_L domains are built from separate genetic elements, and assembled by DNA rearrangements during lymphocyte differentiation. Thus the first and second hypervariable loops are encoded by the germ-line V genes, but the third hypervariable loop by the combination of V, D and J elements (for the V_H domain) and V and J elements (for the V_L domain). The third hypervariable loop of V_H is accordingly the most diverse in sequence and backbone conformation and is often the longest of the loops. Exon structure is illustrated for the unrearranged and rearranged V_H genes and is similar (not shown) for V_L genes ($V\kappa$ or $V\lambda$). V_H, unrearranged or rearranged heavy chain variable region as appropriate; D, D segment; J, J segment; C_H1, C_H2, C_H3, first, second and third heavy chain constant exons, respectively; h, hinge exon; L, signal sequence; p, immunoglobulin promoter (octanucleotide motif). The potential diversity of the mouse primary repertoire is huge as a consequence of the combinatorial arrangements of the genetic elements. It can be estimated as follows. (1) Diversity due to combinatorial integration: $300\ V\kappa \times 4J\kappa = 1.2 \times 10^3$ and $200\ V_H \times 15\ D \times 4J = 12 \times 10^3$. (2) Diversity due to junctional alternatives (alternative readings at junction due to differences in reading frame, and lengths of J and D segments): $V\kappa$ to $J\kappa \sim 3$ and V_H to D to $J \sim 40$. (3) Diversity due to N segment for heavy chain >10. (4) Hence combined diversity for each chain: light chains $= 3 \times 10^3$ and heavy chains $> 5 \times 10^6$. Conclusion: diversity due to combinatorial association of heavy and light chains $> 10^{10}$. Additional factor due to somatic mutation $> 10^{30}$ (see text).

TABLE 1 Immortalization of antibodies using unselected cells from hyperimmunized animals

	Hybridomas	EBV transformation	Random combinatorial gene cloning (bacteria)
Species restrictions	Yes	Only human	No
Chain pairs	H and L	H and L	H and L separate
Positive/negative clones	1/50	1/500(?)	$<1/10^4$ ('artificial')* $<1/10^8$ ('original')
Assay on plates (number of clones as replicas or overlays per plate)	~300	ND	$\sim 10^{3-5}$(?)
Assays on supernatants	Well established, very sensitive	Well established, less sensitive	Possible
Doubling time of cells	18 h	~40 h	30 min
Stability	Good	Poor	Very good
Product	Full molecules	Full molecules	Fragments
Yield of product	20–100 μg ml^{-1}	0.5–5 μg ml^{-1}	0.5–10 μg ml^{-1}

* These values are calculated as follows. If P is probability of finding a particular combination of light (L) and heavy (H) chains in an N-size combinatorial library, and P_L and P_H are the probabilities of random occurrence of each component, $P = 1 - e^{-q}$ where q is NP_LP_H. If we assume that 1% of the mRNA molecules encode antigen-specific immunoglobulin ($P_L = P_H = 10^{-2}$), there is ~60% probability that $1/10^4$ recombinants will contain one H and one L chain encoding any antigen-specific immunoglobulin: most are comprised of artificial combinations, which may or may not specify antigen-binding activity. If there is a dominant lymphocyte clone which represents 1% of the antigen-specific immunoglobulin lymphocytes, the frequency of each chain is 10^{-4} and testing 10^8 clones will give us a 60% probability of finding the 'original' combination. But regardless of whether they originate from antigen-binding lymphocytes, L chains may be capable of complementing the H chains from antigen-specific immunoglobulin. This frequency may be high particularly in primary responses and early secondary responses, before extensive diversification through hypermutation, but will depend on the cut-off affinity used in the assay. Taking a value of 1:100 (ref. 69), there is a 60% chance that $\sim 10^4$ of the recombinants will give an 'artificial' positive signal. EBV, Epstein–Barr virus.

proliferation of cells drawn from the repertoire already available at the time of immunization. The potential repertoire is huge ($>10^{10}$) (Fig. 3 and legend), but at any given time, only a fraction of the potential repertoire of a mouse is available through the limited number of clones (10^7–10^8) expressing antibodies.

The second stage produces high-affinity antibodies, starting with the genes used in the first stage. Its main tool is the hypermutation of these genes followed by selection of those cells which produce antibodies of increased affinity. This is a darwinian process, involving variation resulting from point mutations followed by selection driven by the requirement for antigen for cell survival. It is an inheritable process, but only at the somatic level among the lymphoid cells of the individual. The resulting memory cells seem to be able to undergo new rounds of hypermutation and selection following antigenic challenge[61].

The rate of mutation approaches 10^{-3} or 3×10^{-4} mutations per base pair per cell division in a more recent estimate[60,61,62]. Mutations are localized to the segment coding for the variable portion of the antibody genes. The potential diversity generated by hypermutation is astronomical. Even if only the hypervariable region (~30 residues) of a single antibody variable domain could mutate into 10 out of the 20 amino acids, this would produce about 10^{30} variants for each chain. So the problem at this stage is not the generation of diversity, but the continuous selection of improvements against a background of degeneracy[63]. As immunization proceeds, additional high-affinity antibodies gradually emerge with V–D–J combinations which are rarely found in the primary repertoire (repertoire shift)[64].

In 'classical' antibody engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged antibody VH and VL genes. However, using 'universal' primers and the polymerase chain reaction (PCR) it is possible to rescue V genes[21,23,65,66] and by building restriction sites into these primers the amplified DNA can be cloned directly for expression in mammalian cells[21] or bacteria[23,24].

This offers new routes for the derivation of monoclonal antibody-producing cell lines (Fig. 5). At its simplest, it allows V genes to be rescued from hybridomas[21], unstable hybridoma fusions (for example mouse–human hybridomas), single hybridoma cells[67,68] or even single B lymphocytes. Hybridomas have advantages because fusion enriches for antigen-stimulated cells. However, rescue from single B lymphocytes bypasses cell fusion, allowing access to terminally differentiated B cells which are rich in messenger RNA but are not able to fuse. Single hybridomas or B cells might also be isolated by binding to antigen, immobilized on solid supports (for example on plates or magnetic beads), by fluorescence-activated cell sorting (FACS) or by rosetting with antigen-coated red cells. The single set of V genes from individual cells could then be co-expressed in bacteria. But in this case each cell must be processed separately.

Cloning from heterogeneous cell populations has the advantage that all the cells can be processed together. For example,

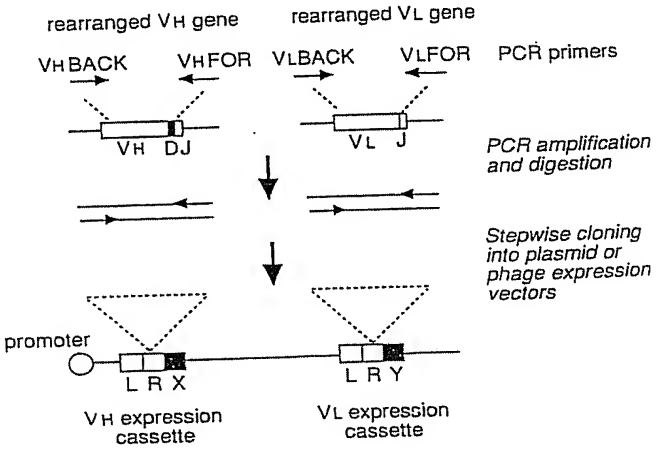


FIG. 4 PCR cloning of rearranged VH and VL genes into expression vectors. Primer mixtures can be designed for PCR amplification of most families of V genes, as the nucleotide sequences at the 5′ and 3′ ends of the rearranged V genes are relatively conserved[21]. At the 5′ end, mixed PCR primers have been based within the signal sequence[65,67] or at the N-terminal end of the mature variable domain[21,66]. At the 3′ end, PCR primers have been located within the J region or the C region. One set of PCR primers, located entirely within the rearranged V-gene exon, amplifies both mRNA and chromosomal DNA[21,23]. The PCR primers incorporate restriction sites and after amplification and digestion with restriction enzymes can be cloned into specialized plasmid or λ phage vectors directly for expression of the V genes. The cloning of VH and VL genes into a plasmid expression vector is illustrated here. (L, leader sequence for secretion into bacterial periplasm and/or beyond outer membrane; R, polylinker cloning site; X and Y, extra polypeptide sequence (for example, heavy chain CH1 domain, light chain CL domain or peptide tag for recognition by monoclonal antibody).)

V_H and V_L genes, taken from total unfractionated cells from an immunized mouse have been combined at random and Fab fragments expressed from λ phage screened for antigen-binding activities[24]. The disadvantage is that the original V_H and V_L pairing, selected for high affinity by immunization, is lost. A combinatorial library with only 1,000 different V_H and 1,000 different V_L gene elements equally represented, would necessitate the screening of between 10^6 and 5×10^6 clones to recover most of the original pairings. Thus the chances of recovering original pairs of V genes from a large random combinatorial library from an immunized mouse are low, and even more remote for the highest affinity antibodies of hyperimmune animals. Those pairs of V_H and V_L domains that are found to be capable of binding antigen[69,70] are likely to do so at lower affinity or with lower specificity than those selected by antigen. Although isolation of an anti-hapten Fab fragment with a good affinity has been reported[24] using such an approach, if the aim of this technology is to bypass hybridomas, it will need to make the high-affinity antibodies required for diagnosis and therapy, antibodies which are difficult to derive even with hybridomas.

To improve the chances of recovering original pairs, the complexity of the combinatorial libraries could be reduced by using small populations of antigen-selected B lymphocytes (Fig. 5). But all combinatorial approaches will rely heavily on powerful screening methods. Here the use of membrane filters for screening large numbers of clones is promising. A variety of formats have been deployed; for example, capture of antibodies on filters coated with antigen, and detection with anti-globulin reagents[10]. Alternatively, antibodies or Fab fragments have been immobilized directly on membrane filters[24] or indirectly through antiglobulin reagents (A. Skerra, M. Dreher, E. Gheradi, G.W. and C.M., unpublished results) and probed with labelled antigen.

Bypassing animals?

Looking ahead, it may become possible to build antibodies from first principles, taking advantage of the structural framework on which the antigen-binding loops fold. In general these loops have a limited number of conformations which generate an endless variety of binding sites by alternative combinations and by diverse side chains[71,72,64]. Recent successes in modelling antigen-binding sites[41,72] augurs well for *de novo* design. This approach might become attractive for making catalytic antibodies, particularly for small substrates. Here side chains or binding sites for prosthetic groups[73] might be introduced, not only to bind selectively to the transition state of the substrate, but also to participate directly in bond making and breaking. The only question is whether the antibody architecture, specialized for binding, is the best starting point for building catalysts. Genuine enzyme architectures, such as the TIM barrel, might be more suitable. Like antibodies, TIM enzymes also have a framework structure (a barrel of β strands and α helices) and loops to bind substrate. Many enzymes with a diversity of catalytic properties are based on this architecture[74] and the loops might be manipulated independently of the frameworks[75] for design of new catalytic and binding properties.

Instead of the 'design and build' approach, could we build an artificial selection system, for example harnessing bacteria or phage, to select for antigen-binding activities? Here no structural information about the antigen is needed. But we see no future in trying to select high-affinity binding activities in a single step. The strategy of the immune system, in which low affinity evolves to high affinity seems more realistic[25]. Can we imitate this strategy and indeed improve on it?

Our first task is to prepare a naive repertoire of antibody genes (Fig. 6). At its simplest we could use the polymerase chain reaction (PCR) and universal primers to reproduce *in vitro* the repertoire of rearranged V genes expressed by naive animals. However 'naive' animals are not really naive and the available repertoire is limited in size and shaped, for example by tolerance to self-epitopes. In principle we could adopt the mammalian strategy of assembling a much larger repertoire by random combination between restriction fragments encoding the germline V, D and J elements. To match the potential repertoire of the animal, we would also have to reproduce the junctional diversity created by recombination. Large repertoires might also be made by trying to immitate the process of gene conversion adopted by birds[76].

But such 'natural' repertoires are not ideal. The sequences of germ-line V genes and the multiplicity of highly related V genes in the genome are presumably themselves the result of chance and evolutionary pressures, for example towards recognition of pathogens and against recognition of self-components. Such repertoires are both biased and highly redundant. More efficient repertoires might be constructed by using V genes from a variety of animal sources, excluding highly related V genes and even designing entirely new V genes or D segments. For example, the structures of individual antibody loops are often very similar

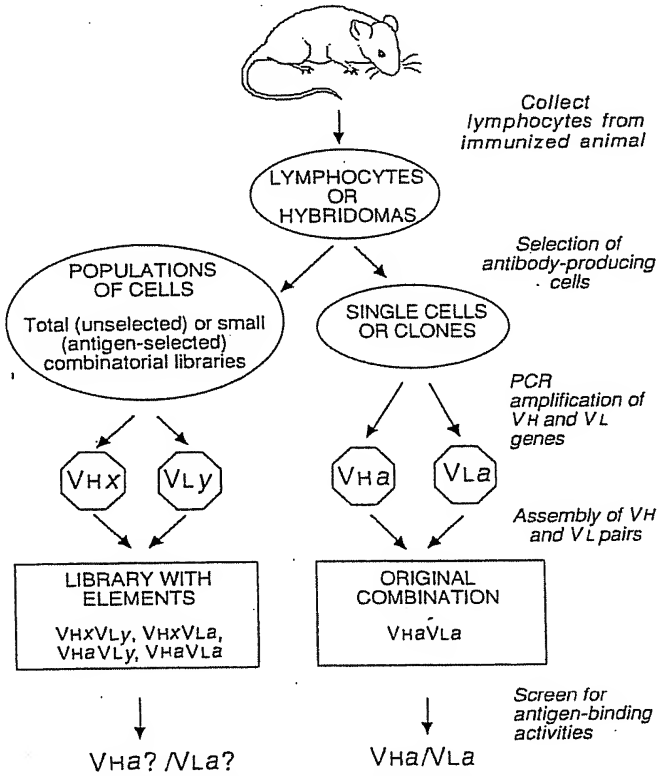


FIG. 5 Strategies for cloning paired V_H and V_L genes from lymphocytes of an immunized animal. V_H and V_L genes (expressed as pairs V_Hx/V_Ly) from n lymphocytes can be amplified by PCR from the mRNA or genomic DNA of single cells or populations. The lymphocytes can also be selected by antigen-binding, or immortalized as hybridomas by fusion with myeloma cells or by infection with Epstein–Barr virus. From single lymphocytes, or clones, the original V_Ha/V_La gene combination is readily rescued. From populations of lymphocytes, repertoires of V_H–V_L genes (V_Ln) could be combined at random, and antigen-binding combinations selected. But for large populations of lymphocytes, original combinations of V_H and V_L genes will be a minor proportion of heterologous combinations, some of which may display residual antigen-binding activity. The library will contain $(n-1)^2$ V_HxV_Ly elements, $(n-1)$ each of V_HxV_La and V_HaV_Ly elements and a single V_HaV_La element. V_Ha and V_La (or close derivatives), however, may occur repeatedly. Thus, at earlier stages or immunization, there are likely to be some artificial pairs which originate from different but closely related clones which can bind antigen, particularly with responses dominated by 'unique' V_H and V_L gene combinations (restricted or idiotypic responses[59,60]). Ref. 90 is in agreement with this prediction. As maturation for high affinity proceeds, the number of mutations increase and the likelihood of effective heterologous complementation diminishes.

in their overall fold[71,72], and the loops could be fleshed with diverse side chains.

The next stage is to express the library and screen for antigen-binding activities by random combination of VH and VL domains. Ideally the library should be 'complete', containing antibodies of a minimum binding affinity for any conceivable epitope. The tighter the binding of a primary antibody, the larger the library required. For example, it has been estimated that a primary repertoire of 10^7 different antibodies is likely to recognize more than 99 per cent of epitopes with an affinity constant of 10^5 M^{-1} or better, and rarely contributes high-affinity antibodies to an epitope taken at random ($>10^9$ M^{-1}) (ref. 77). If millions of VH and VL combinations from an artificial library could be screened, for example on membrane filters with a cut-off affinity of at least 10^5 M^{-1}, then the library would be as complete as the primary repertoire of a single mouse (10^7 antibody species). In the future, the screening of large libraries may well be replaced by methods of selection. For example, by expressing functional antibody fragments at the surface of phage, desired V-gene combinations can be enriched by binding to antigen[78]. If these screening and selection techniques worked as well as those in the animal, we could imitate the primary response and generally obtain low-affinity antibodies.

The enhancement of affinity *in vivo* can be contributed by a single point mutation, or several mutations[64]. Hypermutation of the genes corresponding to antibodies of low affinity should be easy to imitate *in vitro*. Point mutations could be introduced into the V genes by many techniques, for example, by using error-prone polymerases, PCR amplification through a large number of cycles, biased ratios of nucleotide triphosphates or 'spiked' oligonucleotide primers. Multiple mutations could be targeted throughout the body of the gene simultaneously, or to each of the hypervariable loops[79].

The screening or selection of the mutants with improved affinity is likely to be more difficult, as it needs to discriminate between mutants differing slightly in affinity. Such discrimination is possible for hybridoma clones in agarose[10], by binding secreted antibody on coated membranes with different antigen density. The differential binding to antigen-coated membranes might also be achieved by competiton with low-affinity antibody. The system we are proposing, however, is primitive compared with the animal. Ideally we would like to hypermutate specific segments of DNA within cells rather than in isolated DNA, and at the same time express the products on the cell surface, and select in a darwinian fashion variants of steadily increasing affinity. Animals are superb at this job, and it will not be easy to compete with their efficiency. We may learn to imitate the animal strategy, but in the meantime for the production of high-affinity antibodies we will normally do better by stealing hypermutated V genes from immunized animals.

Human antibodies: today and tomorrow

Making human monoclonal antibodies has posed difficulties for hybridoma technology. For example, the use of the mouse myeloma as a fusion partner for human cells leads to preferential loss of human chromosomes, and intolerable instability of the hybrids. Hybridomas are derived from spleen or lymph nodes, whereas the primary source from humans, the peripheral blood lymphocytes, contains few blast cells actively involved in the immune response. As an alternative to fusion, immortalization of human cells by Epstein–Barr virus does not lead to preferential immortalization of blasts engaged in antibody responses, and also leads to lines which are low producers of antibody and unstable[80]. Further, humans can rarely be hyperimmunized to order, especially with noxious chemicals, pathogenic viruses or cancer cells. In any case, the isolation of human antibodies to human cell-surface antigens would have to overcome tolerance mechanisms which eliminate lymphocytes with self-reactivity. Human lymphocytes have recently been used to populate severe combined immunodeficient (SCID) mice and these animals can be immunized[81,82] to make human antibodies.

Gene technology offers alternatives. The 'humanizing' of rodent monoclonal antibodies is currently the most practical approach. It allows access to a vast pool of rodent antibodies with good affinities and specificities. This is a major advantage, particularly when dealing with antibodies for tumour therapy, or for the manipulation *in vivo* of the human immune system. Thousands of antibodies have already been made against human

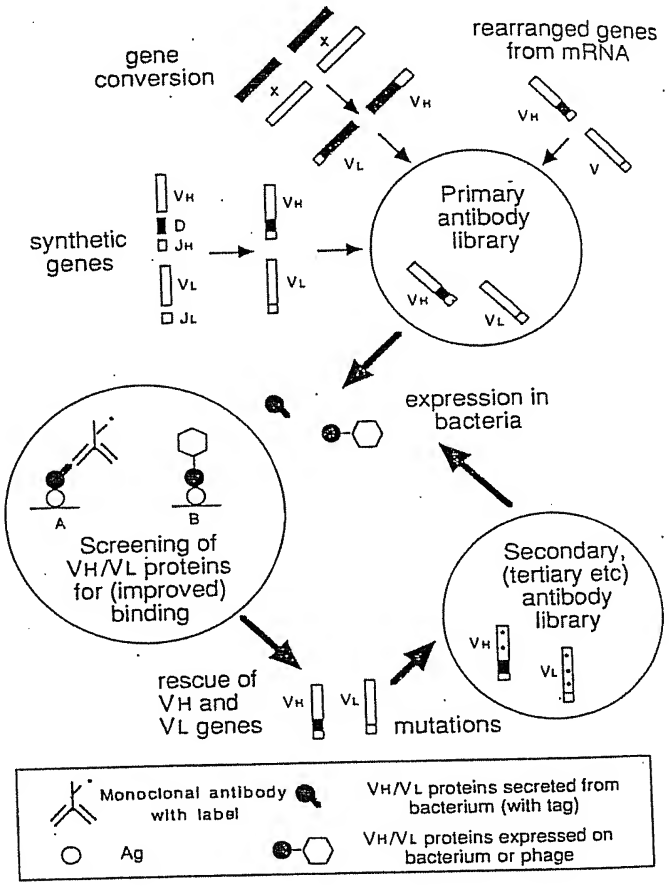


FIG. 6 Mimicking the immune system. VH and VL gene repertoires derived by a range of methods, such as reproduction of the available library from lymphoid cells, assembly of V, D and J elements, or gene cross over (to mimic gene conversion). The genes are cloned and expressed, and VH–VL pairs binding to antigen can be selected or screened, for example on membrane filters. Here the VH–VL pair, binding to antigen (Ag), is tagged with a C-terminal peptide recognized by a monoclonal antibody (mAb), or is expressed on the surface of a bacterium or phage. For detection, the mAb or Ag can, for example, be coupled to radioisotope, or to an enzyme for production of a coloured dye. A, Capture of VH–VL with antigen and detection with mAb. Other schemes could involve capture by binding to membrane and detection with Ag or capture by mAb and detection with Ag. B, Capture with Ag and detection of phage plaques. The genes encoding VH–VL pairs, identified as antigen-binding, can be rescued and hypermutated to form the secondary antibody libarary. These genes can then be expressed and screened for improved binding to antigen. This process will need to be repeated several times to achieve the efficiency of animal immune systems. Selection systems for catalytic antibodies might differ from those above. Such antibodies have been made through hybridoma technology by immunization with transition-state analogues, or by chance[2,3]. But animals select antibodies on the basis of binding, not catalysis, and the transfer of V-gene repertoires to bacteria gives a new twist to the field[24]. Vast libraries might be screened directly, not for binding, but for catalysis for example with substrates yielding fluorescent products, or by complementation of bacterial mutants deficient in a catalytic step of interest. In this case, antibodies will need to be expressed intracellularly[89].

cell-surface antigens, and particularly against human leukocytes[4]. Reshaping these antibodies, by transplanting only the antigen-binding loops to human antibodies[29,31], yields humanized antibodies which may have similar immunogenicity to truly human antibodies. In future, several other approaches may become available through gene technology as discussed in earlier sections. Furthermore, transgenic mice have been made which carry immunoglobulin heavy V, D, J and human C regions[83] and this should allow human antibodies to be produced directly from hyperimmunized mice. It remains to be seen whether the size of the repertoires, limited by the amount of new DNA that can be carried by the transgenic animal, will be a drawback.

But all these methods will have to compete with immortalization by Epstein–Barr virus and cell fusion, which themselves are constantly improving, particularly as they start to incorporate ideas and techniques involving DNA manipulations. There are immune responses which are unique to humans, or which need to be characterized in humans, including those to antibodies in certain auto-immune, parasitic or infectious diseases, or cancer. Here the rescue by gene technology of human hybridomas or cell lines immortalized by Epstein–Barr virus may provide a powerful way of defining the properties of such antibodies and immortalizing them[68].

As Ehrlich wrote 90 years ago[84], '... we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics'. We see a jungle of technologies, old and new, stimulating each other: in the immediate future, most of them start with immunized animals. □

Greg Winter and Cèsar Milstein are in the MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

1. Köhler, G. & Milstein, C. *Nature* **256**, 52–53 (1975).
2. Tramontano, A., Janda, K. D. & Lerner, R. A. *Science* **234**, 1566–1569 (1986).
3. Pollack, S. J., Jacobs, J. W. & Schultz, P. G. *Science* **234**, 1570–1573 (1986).
4. Knapp, W. *et al.* (eds) *Leucocyte Typing IV: White Cell Differentiation Antigens* (Oxford Univ. Press, Oxford, 1989).
5. Rodwell, J. D. & McKearn, T. J. *Biotechnology* **3**, 889–894 (1985).
6. Vitetta, E. S. & Uhr, J. W. *A. Rev. Immun.* **3**, 197–212 (1985).
7. Bagshaw, K. D. *Br. J. Cancer* **56**, 531–532 (1987).
8. Ekins, R. *Nature* **340**, 256–258 (1989).
9. Casali, P., Inghirami, G., Nakamura, M., Davies, T. F. & Notkins, A. L. *Science* **234**, 476–479 (1986).
10. Gheradi, E., Pannell, R. & Milstein, C. *J. Immunol. Meth.* **126**, 61–68 (1990).
11. Rudikoff, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1979–1983 (1982).
12. Brüggemann, M., Radbruch, A. & Rajewsky, K. *EMBO J.* **1**, 629–634 (1982).
13. Radbruch, A. *Handbk exp. Immun.* **4**, 110.2–12 (Blackwell Scientific Publishers, Oxford, 1986).
14. Milstein, C. & Cuello, A. C. *Nature* **305**, 537–540 (1983).
15. Oi, V. T. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 825–829 (1983).
16. Neuberger, M. S. *EMBO J.* **2**, 1373–1378 (1983).
17. Cabilly, S. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 3273–3277 (1984).
18. Boss, M. A. *et al. Nucleic Acids Res.* **12**, 3791–3806 (1984).
19. Skerra, A. & Plückthun, A. *Science* **240**, 1038–1040 (1988).
20. Better, M. *et al. Science* **240**, 1041–1043 (1988).
21. Orlandi, R. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 3833–3837 (1989).
22. Chiang, Y. L. *et al. BioTechniques* **7**, 360–366 (1989).
23. Ward, E. S. *et al. Nature* **341**, 544–546 (1989).
24. Huse, W. D. *et al. Science* **246**, 1275–1281 (1989).
25. Milstein, C. *Proc. R. Soc.* B **239**, 1–16 (1990).
26. Silverstein, A. M. *A History of Immunology* (Academic, San Diego, 1989).
27. Neuberger, M. S., Williams, G. T. & Fox, R. O. *Nature* **312**, 604–608 (1984).
28. Neuberger, M. S. *et al. Nature* **314**, 268–270 (1985).
29. Jones, P. T. *et al. Nature* **321**, 522–524 (1986).
30. Brüggeman, M. *et al. J. exp. Med.* **166**, 1351–1361 (1987).
31. Riechmann, L. *et al. Nature* **332**, 323–327 (1988).
32. van der Zee, J. S., van Swieten, p. & Aalberse, R. C. *Clin exp. Immun.* **64**, 415–422 (1986).
33. Duncan, A. R. *et al. Nature* **332**, 563–564 (1988).
34. Duncan, A. R. & Winter, G. *Nature* **332**, 738–740 (1988).
35. LoBuglio, A. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 4220–4224 (1989).
36. Brüggemann, M. *et al J. exp. Med.* **170**, 2153–2157 (1989).
37. Kabat, E. A. *et al. Sequences of proteins of immunological interest* (US Department of Health and Human Services, US Government Printing Office, 1987).
38. Verhoeyen, M., Milstein, C. & Winter, G. *Science* **239**, 1534–1536 (1988).
39. Queen, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 10029–10033 (1989).
40. Hale, G. *et al. Lancet* **ii**, 1394–1399 (1988).
41. Roberts, S., Cheetman, J. C. & Rees, A. R. *Nature* **328**, 731–734 (1987).
42. Suresh, M. R., Cuello, C. & Milstein, C. *Meth. Enzym.* **121**, 210–228 (1986).
43. Lanzavecchia, A. & Scheidegger, D. *E J. Immun.* **131**, (1987).
44. Clark, M. & Waldmann, H. *J. natn., Cancer Inst.* **79**, 1393–1401 (1987).
45. Chaudhary, V. K. *et al Nature* **339**, 394–393 (1989).
46. Schnee, J. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **84**, 6904–6908 (1987).
47. Bagshawe, K. D. *et al. Br. J. Cancer* **58**, 700–703 (1988).
48. Byrn, R. A. *et al. Nature* **344**, 667–670 (1990).
49. Sutherland, R. *et al. Cancer Res.* **47**, 1627–1633 (1987).
50. Covall, D. G. *et al. Cancer Res.* **46**, 3969–3978 (1986).
51. Wenger, T. L. *et al. J. Am. Coll. Cardiol.* **5**, 118–123 (1985).
52. Saul, F. A., Amzel, L. M. & Poljak, R. J. *J. biol. Chem.* **253**, 585–597 (1978).
53. Boulot, G. *et al. J. molec. Biol.* **213**, 617–619 (1990).
54. Riechmann, L., Foote, J. & Winter, G. *J. molec. Biol.* **203**, 825–828 (1988).
55. Cabilly, S. *Gene* **85**, 553–557 (1989).
56. Glockshuber, R., Malia, M., Pfitzinger, I. & Plückthun, A. *Biochemistry* **29**, 1362–1367 (1990).
57. Bird, R. E. *et al. Science* **423**, 423–426 (1988).
58. Huston, J. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 5879–5883 (1988).
59. Griffiths, G. M., Berek, C., Kaartinen, M. & Milstein, C. *Nature* **312**, 271–275 (1984).
60. Kocks, C. & Rajewsky, K. *A. Rev. Immun.* **7**, 537–559 (1989).
61. Berek, C. & Milstein, C. *Immunol. Rev.* **105**, 5–26 (1988).
62. McKean, D. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3180– (1984).
63. Allen, D. *et al. Immunol. Rev.* **96**, 5–22 (1987).
64. Berek, C. & Milstein, C. *Immunol. Rev.* **96**, 23–41 (1987).
65. Larrick, J. W. *et al. Biochem. biophys. Res. Commun.* **160**, 1250–1255 (1989).
66. Sastry, L. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 5728–5732 (1989).
67. Larrick, J. W. *et al. Biotechnology* **7**, 934–938 (1989).
68. Larrick, J. W. *et al. ICSU Short Rep.* **10**, 93 (IRL Press 1990).
69. Zhu, D., Lefkovitis, I. & Köhler, G. *J. exp. Med.* **160**, 971–986 (1984).
70. Hudson, N. W. *et al. J. Immun.* **139**, 2715–2723 (1987).
71. de la Paz, P., Sutton, B. J., Darsley, M J. & Rees, A. R. *EMBO J.* **5**, 415–425 (1986).
72. Chothia, C. *et al. Nature* **342**, 877–883 (1989).
73. Baldwin, E. & Schulz, P. G. *Science* **245**, 1104–1107 (1989).
74. Chothia, C. *Nature* **333**, 598–599 (1988).
75. Luger, K. *et al. Science* **243**, 206–209 (1989).
76. Reynaud, C. *et al.* in *Immunoglobulin Genes*, 151–162 (Academic, London, 1989).
77. Perelson, A. S. *Immunol. Rev.* **110**, 5–33 (1989).
78. McCafferty, J., Griffiths, A. D., Winter, G. & Chiswell, D. J. *Nature* **348**, 552–554 (1990).
79. Ward, E. S. *et al. Prog. Immun.* **7**, 1144–1151 (Springer, Berlin, 1989).
80. Roder, J. C., Cole, S. P. C. & Kozbor, D. *Meth. Ezym.* **121**, 140–167 (1986).
81. Mosier, D. E. *et al. Nature* **335**, 257 (1988).
82. McCune, J. M. *et al. Science* **241**, 1632 (1988).
83. Brüggemann, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 6709–6713 (1989).
84. Ehrlich, P. *Proc. R. Soc.* **66**, 424–448 (1900).
85. Marquart, M., Deisenhofer, J. & Huber, R. *J. molec. Biol.* **141**, 369–391 (1980).
86. Lesk, A. M. & Hardman, K. *Science* **216**, 539–540 (1982).
87. Taub, R. *et al. J. biol. Chem.* **264**, 259–265 (1989).
88. Williams, W. V. *et al. Proc. natn. Acad. Sci. U.S.A.* **86**, 5537–5541 (1989).
89. Carlson, J. R. *Molec. cell. biol.* **8**, 2638–2646 (1988).
90. Caton, A. J. & Koprowski, H. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6450–6454 (1990).

ACKNOWLEDGEMENTS. We thank A. Lesk and S. Pledger for help in preparing Fig. 1, and our colleagues for advice.